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STUDIES ON MELANIN

(REPORT II)

By

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PREFACE

In 1952, I published a compilation of the results of my previous studies on skin pigment in Tohoku J. Exp. Med., Vol. 55, Supplement I, under the title " Studies on Melanin ", and read a summary of the same at the Tenth International Congress of Dermatology held in London (1952). The data obtained thereafter were published as a prearranged report at the 55th Annual Meeting of Japanese Dermatological Association at Sendai in May 1956. At that meeting, I feel it my pleasant duty to add, Prof. T. B. Fitzpatrick, University of Oregon, who had come all the way from Portland, gave a special lecture on " The Problem of Melanin Formation ", and many dermatologists from all parts of Japan made edifying reports and exhibits on the occasion, giving a splendid success to the meeting where the central theme of discussion was limited to the problem of melanin.

In this volume I have published the details of my report at the Annual Meeting under the title " Studies on Melanin, Report II ", in which I have noted further particulars on my hitherto expressed opinions in full, believing that I have succeeded in presenting some significant data concerning the question of melanin formation based on many new findings of our research team in Tohoku University. All the histological, histochemical, hematochemical and endorcinological observations and experiments presented in this volume have always been made with the change of melanin content in the human body as their objective. For reference' sake, the Contents and General Conclusion of my Report I are reprinted as an appendix at the end of this volume.

I hereby express my heartiest thanks due to Drs. Iijima, Iisawa, Ishitoya, Kamada, Komatsu, Kurosaki, Noguchi, Saito, Sato, Shimoda, Suzuki, Takahashi, Tanita, Tanji, Togashi, Tokunaga, Tsukada, Usuba, Yamabe and Yoshida for their helpful cooperation in my research.

Sendai
February, 1957

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I. Histological Investigations on Epidermal Melanin-Forming Cells

The dendritic cells in the human epidermis was first pointed out by Langerhans' gold-impregnation in 1868, and the presence of dendritic cells containing melanin was made known by Riehl in 1884. Later, in 1917, Bloch in his dopa reaction found that the positively reacting cells were of the dendritic form, and more recently, Fitzpatrick, Lerner *et al.*[1] also pointed out that the cells which, activated by ultraviolet irradiation, gave a positive tyrosinase reaction showed a similar dendritic form. Thus it has been confirmed that these cells are made visible through reduction by gold or silver impregnation or through oxidation by dopa-oxidase or tyrosinase, but as to their essential nature, opinions have been divided to date, all repeating their being identical with or different from nerve terminations or melanin-forming cells. As early as 1946, I[2] gave a description of the problem of nevus cells. I have not tried Langerhans' gold-impregnation method, but by Bielschowsky-Seto's silver impregnation I have often observed figures closely resembling the so-called Langerhans' cells, as in John's report. By Bezecny's iron-alum-hematoxylin staining I have also stained figures representing Langerhans' cells and dendritic cells in the basal layer of the epidermis, demonstrating that these two kinds of cells are of an entirely identical shape. The findings obtained by Ferreira-Marques on the negative result of Langerhans' cells in dopa reaction were confirmed in my preparations subjected to dopa and tyrosinase reactions. Thus I am of the opinion that Masson's interpretation of Langerhans' cells in wellfounded. Here I will discuss only the melanin-forming cells distributed in the basal layer of the epidermis.

Experiments and Results

When examining the sacral skin of fetuses and new-borns by Bielschowsky-Seto's method, I noticed the appearance of dendritic cells in the epidermal basal layer in a specimen of fetus of only six's month. They were argentophil cells presenting their longer axes horizontally among somewhat pallisade-like arranged basal cells, each sending out several dendritic processes. They apparently protruded somewhat inside the corium beyond the epidermodermal junction, and sometimes two very similar cells, lying one upon another, looked as if they were astride the junction, presenting the figure of the so-called twin cells. They were arranged at somewhat regular intervals. Thus, I have found in the speci-

men of fetus of six's month a type of cells showing a considerably dissimilar appearance to that of the basal cells arranged in a pallisade. When the same specimen was stained with hematoxylin-eosin, however, no dendritic processes were observed at all, only I could surmise that the cells with their nucleus apparently floating amidst a limpid cytoplasm sporadically among the basal junction were the above-said dendritic cells.

In skin specimens from an eight's month fetus, silver-reducing granules just made appearance throughout the basal layer so that under low magnification the layer was distinguished as a darker zone from the lighter upper layer. In fetuses of older age or newborns the longer axes of the dendritic cells were observed to turn more perpendicular to the junction of the basal layer, thus tending to become aligned with the basal cells in general, but their density of distribution showed little increase, so that with the extension of the skin area, their intervals seemed to elongate. This seems to suggest that the dendritic cells remain unincreased in number since their differentiation at a rather early stage of embryonal life.

Similar examinations of skin slices from foci of intraepidermal nevus, nevus-cell nevus, ephelides inversae, and acropigmentatio reticularis Kitamura, and from the papillae of infants and the scrota of human adults revealed that the darkpigmented sites of skin contained dendritic cells in high density. This is quite natural in the prenatally abnormal sites which are dark above the ordinary physiological tone, and in particular, this correlation between the density of pigmentation and that of the distribution of dendritic cells is remarkable in such localized hyperpigmented foci as found in ephelides inversae cases. In a lentigo achromiant of congenital albino recently discovered by us[3], the so-called cellules claires were, due to lack of melanin, demonstrated more distinctly than usual, what Masson calls thèques were found to be formed of such cellules claires, and dendritic cells were observed under iron-alum-hematoxylin staining.

The dopa-positive cells in the skin slices from the abovementioned specimens which had been simultaneously subjected to dopa reaction also showed similarity in their findings as to their formation, location and distribution to those observed under Bielschowsky-Seto's silver impregnation, the difference being very slight. The pictures of these cells are also nearly identical with those of the cells reacting positively in the tyrosinase reaction of skin slices from the human armpit and thigh which were conducted by the original Fitzpatrick's method or its cupric sulfate modification[4]. Recently, Zimmermann and Cornbleet[5], who made examinations of the skin from Negro fetuses, reported that the positive dopa reaction of the dendritic cells in the basal layer first appeared in the 3–4th months of the fetal life; and that in the 4–5th months granules of melanin were found in such cells in unstained skin slices. Their discovery

of the dendritic cells in somewhat earlier stages of fetal life compared with our discovery described above may be due to the racial difference in color between the fetuses.

Comment

On the origin of the melanin-forming dendritic cells, the Bloch school have interpreted, based on the findings in dopa reaction, that these cells are different from Langerhans' cells in origin, basal cells presenting dendritic forms according to their changed functions. Miescher[6], Peck[7], and Ormsby and Montgomery[8] also presumed transition from basal cells, and we too used to agree with this view. More recently, however, the view that the dendritic and the basal cells are of different origins, supported by the embryological studies of amphibians and birds, has gained such an influence that even Miescher and Montgomery have come to modify their opinions. The specificity in form and location of these cells demonstrated throughout our above-described observations from the fetal period has led us to admit their being different from the common basal cells.

As to the nature of these cells, Kyrle, Pautrier and Masson[9] identified them with Langerhans' cells, Becker, Jr., Fitzpatrick and Montgomery assumed that the dopa-positive cells were akin to Langerhans' cells, and John[10] reported on his success in staining Langerhans' cells by Bielschowsky-Gross' staining method. Ferreira-Marques[11] presumed Langerhans' cells to be Schwann's cells wandering out into the epidermis. Masson (1948) identified the melanogenous dendritic cells with cellules claires corresponding to Merkel's touch cells, postulating their nervous origin. Rawles[12], Becker[13] and Pinkus adopted the neural-crest theory on their origin, and Kawamura and his collaborators[14] also supported it upon their own through going studies. We are in concurrence with the nerve theory of Masson who advocates the identity of the nevus cells with Schwann's nuclei in origin, but on the other hand, we are of the opinion, as are Ranvier, Cajal, Martinetz Pérez, Rodriguez Pérez, Foerster, Kadanoff, Weddell and Seto, that no nerves are found in the adult human epidermis, considering the results of our examinations of many silver-impregnated preparations. Thus, from the above observations, we are led to infer that melanogenous dendritic cells are cells which have acquired their specific nature and function by the differentiation of the peripheral nerve fiber after it has penetrated into the epidermis with an embryological aim of attaining the skin surface in the fetal stage, in other words they are, in our interpretation, of the neuro-epidermal origin in its broad sense. Moreover, as Nishihara[15] has already pointed out, we have often encountered nerve fibers or nerve terminal reticula immediately beneath

such dendritic cells. This and the fact that ephelides inversae occur only in the parts rich in Meissner's touch corpuscles and other terminal nerve organs, such as the lip, palm and sole, with densely located melanin-forming dendritic cells demonstrable in the lesions seem to justify the probable interrelationship between these cells and the nerves.

As to the relationship between the appearance of melanin granules and the basal cells in general, it has at last been generally accepted since the report by Becker that the dopapositive cells are all dendritic cells. Recently, Becker, Fitzpatrick and Montgomery[16], based on the results of dopa reaction tests of excised epidermis prepared with pancreatin by methods similar to those of Medawar and Bellingham, have propounded a novel theory that the dendritic processes of such cells activated by thorium X are sent out widely into the surrounding tissues, sometimes mutually anastomosing to form melanin retes, and that the basal cells in general contain no granules of melanin. Besides, Becker, Jr. suggests the term melanodendrocyte and Fitzpatrick melanogenocyte. This view of theirs has called up a new interest on the problem, together with their method of application of tyrosinase reaction for estimating the malignancy of melanoma. The view that melanin is supplied to the basal cells from the dendritic cells, however, had previously been published by Vilter[17].

However, the above-stated new proposition by Becker *et al.* as to the physiological conditions of melanin in the epidermis of the human skin, *e.g.*, the distribution of distal nuclear caps, the almost uniform distribution and nature of the granules of melanin both in the dendritic and basal cells and furthermore their view that blank areas of intercellular spaces are clearly demonstrated among the groups of the silver-impregnated granules cannot be readily complied with, and in particular, it may be inappriate to infer the mechanism of physiological melanin formation by the analogy of the experimental results induced by strong irradiation of ultraviolet rays.

In our previous histochemical examinations of the indophenoloxidase reactions (the tissue-Nadi reaction by Graeff) of the cells known as melanogenous cells, namely, dendritic cells in basal layer, nevus cells, Mongolian spot cells, blue nevus cells and malignant melanoma cells, and of the dopa-positive pigment cells discovered by us in nevus Ota and Riehl's melanosis, we, Itô and Watanabe[18], clearly observed blue granules similar to granules of melanin in size and form. Such indophenol-blue granules were found in larger quantities in cells containing little melanin than in those containing much melanin. The results of tissue peroxidase reaction by methods similar to Sato-Sekiya's method or Okano's method were positive in the nuclei of these cells as a rule, and diffuse or granular pigmentation was frequently observed even in the

cytoplasm at the sites where the nuclei of basal cells, nevus cells and dermal melanoblasts in particular showed intense positive reactions. The capillary endothelial cells also showed strong positive oxidase and peroxidase reactions, and positive granules in both reactions were observed to be overflowing into the surrounding tissues. At the same time, we demonstrated, as described in Report I, that the leucocytes in the local blood of the skin with pigmented nevus and of anomal skin with black spots gave stronger peroxidase reactions than those in the control blood taken from normal ear-lobes and that the local blood from the skin with vitiligo showed also a prolongation in time of peroxidase reaction. In our recent peroxidase reaction tests by Sato's quick method[19] in the leucocytes of the periphery blood from 8 cases of complete albinism, we found a marked prolongation in staining time as shown in Table I. Studying these histological and

TABLE I

The Short-timed Peroxidase Reaction of Blood Leucocytes in Complete Albinism (by Sato-Sekiya's Copper Method)

Age (years)	Sex	Shortest stain time (seconds)
1	F	15
3	F	10
3	F	9
8	M	8
1	M	8
8	F	6
1	F	5
20	F	5
Normal control		2–5

hematological findings in collation, we infer that between osteomedullar leucocytes and histiocytes there may be a correlation of giving and taking enzymes through the capillaries or a field for adapting the activity of such enzymes, and wish to emphasize the importance of the nonspecific enzyme oxidase and peroxidase distributed in the pigment cells as relating to the catalysis which participates in the physiological development of melanogenesis. In our previous report we summarized many studies suggestive of the correlation of polyphenolase and peroxidase with melanin formation in the skin tissue.

SUMMARY

Dendritic cells of human epidermis are derived from nervous endorgans which have reached to the epidermis in the embryonal period

and differentiated to reveal specific melanogenic activities. They represent the primary site of melanogenesis.

The distribution of indophenolase and peroxidase activities in the pigment cells suggests that these two enzymes participate in the physiological manifestation of melanogenous activity. The variation of peroxidase activity of leucocytes in the pigmentary disorders supports this conception.

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II. Histochemical Investigations on Epidermal Melanin Formation by Means of Ultraviolet Irradiation

The relationship between human skin color and sunlight or ultraviolet rays has long been discussed. Darwin gave an evolutional explanation of the fact that the skin of black races has a resistence to the tropical climate, Unna confirmed that melanin has a capacity to absorb chemical rays, and Finsen, Jesionek and others emphasized a physiological significance of melanin deposits in the epidermis. Recently, Fitzpatrick and Lerner[1] reported that ultraviolet irradiations activate melanocytes in the human epidermis. In the present experiments, an ultraviolet irradiation was performed on normal human skin and its influences were histochemically investigated.

Experiments and Results

Normal skin of ordinarily covered sites such as the extensor side of the thigh and the axilla was irradiated with artificial sun-light at 30 cm for 20, 40 and 60 minutes, excised 1, 3 and 5 days thereafter and histochemically examined for dopareaction, tyrosinase reaction and changes in the nucleic acids.

1) *Hematoxylin-eosin Staining:* To examine the behavior of epidermal melanin, the granules of pigment in the non-irradiated control samples are found almost exclusively in the basal layer, presenting a dark zone; but granules of pigment gradually appear in the prickle cells of the superficial layer in the skin irradiated for 20–40 minutes; in the skin slices excised 3 days after 60 minutes' irradiation, melanin appears even just beneath the granular layer, showing the appearance of distal nuclear caps wherever they are, the granules of melanin becoming diffusive at the same time, the pictures of the individual cells becoming blurred and somewhat hypertropic, and acanthosis being demonstrated; and in some of the skin slices excised 5 days after 60 minutes' irradiation, necrobiosis is observed in the Malpighian rete. The changes in the corium represent dilatation of the blood vessel and cellular infiltration (chiefly round cells) in proportion to the length of irradiation.

2) *Dopa Reaction:* The reaction is increased even in the skin slices excised 3 days after 40 minutes' irradiation; in the samples 3 days after 60 minutes' irradiation, the rise of all the processes from the dendritic cells in the basal layer into the upper layer is so remarkable that they

appear to cover up the lower half of the prickle-cell layer an appearance reminding us of the picture magnified melanogenocytes obtained by Fitzpatrick in his experimental irradiation.

3) *Tyrosinase Reaction Tests by Methods Similar to Those of Fitzpatrick:* Skin slices 3 days after 40–60 minutes' irradiation show slightly positive reactions in the dendritic cells of the basal layer, and skin slices 5 days after irradiation show somewhat distinctly positive reactions in the dendritic cells, but those 7 days after irradiation show no positive reactions any longer.

4) *Nucleic Acid Staining:* Unna-Pappenheim's method, thionine staining, Feulgen method and Erickson's perchloric acid treatment were applied. In the non-irradiated skin slices, pyronin-phil nucleoli are distinctly seen all over the Malpighian layer (Fig. 1). The skin slices one day after 20 minutes' irradiation show hardly any change. But in the specimens 3–5 days after irradiation the nucleoli of the cells in the basal layer are rendered more distinct by pyronin staining and cells with slightly increased melanin are sporadically observed, probably representing melanogenous dendritic cells. In the skin slices 1–3 days after 40 minutes' irradiation, the nucleoli of the cells in the Malpighian layer are seen to be gradually increased in size and color, in some cases 2–3 nucleoli are coexisting in one nucleus and in others nucleoli appear to be wandering away from the nucleus and the granules of melanin are gradually developing diffusively upward. In the skin slices excised 3–5 days after 60 minutes' irradiation, granules of melanin in the form of nuclear caps are seen close to the upper poles of the nuclei containing enlarged, strongly pyronin-stained nucleoli in the prickle-cell layer near the granular layer (Figs. 2, 3). In the specimens treated by Erickson method, nuclear-cap-like melanin deposits can be distinctly demonstrated because of the fading of RNA (Fig. 4). The cytoplasmatic RNA are found slightly increased in the 20 minutes irradiated specimens, but in the slices of skin irradiated 40–60 minutes the acid appears somewhat to decrease from the onset, in some cases vacuolation being observed. With the lengthening of irradiation, the nucleic DNA gradually decreases or comes to be coagulated in striae in the vicinity of the nuclear membrane. Such kinetic behaviors of the nucleic acids in the epidermic layer were also observed in our separately conducted experiments by means of various chemical stimulations[2], but such a marked mobilization of melanin as found in these experiments were hardly ever observed in those others.

Comment

This finding on the growth of nucleoli is in a way related with the description by Meirowsky[3] that melanogenesis is preceded by an increase

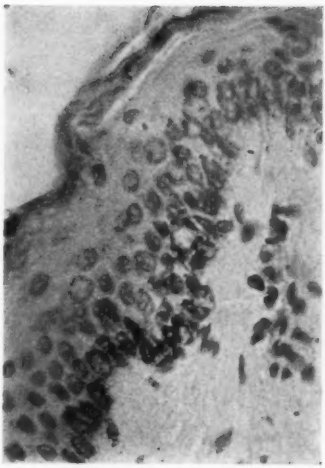

Fig. 1. Unirradiated human skin (Unna-Pappenheim's stain).

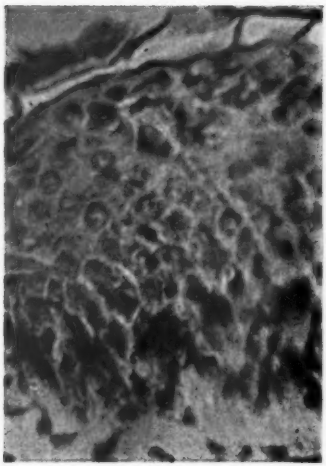

Fig. 2. Three days after 60 minutes' ultraviolet irradiation (Unna-Pappenheim's stain).

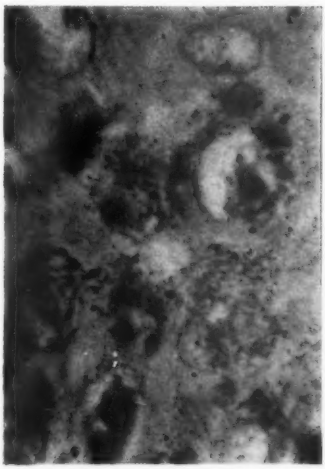

Fig. 3. Oil immersion ofFig. 2.

Fig. 4. Same specimen as Fig. 2 treated by Erickson method.

in a pyronine-red stained substance in the nuclei, and that this substance, upon reaching the cytoplasm, becomes the mother substance of melanin. Meirowsky at first proposed this to disprove Bloch and his followers' theory of extranuclear mother substance of melanin. More recently, he has taken up the nucleic-acid suggestion by Casperson and others, and making reference to the report by DuBuy, Wood, Burk and Lackey that the melanin in melanoma of S-91 mice contains about 20 per cent of nucleic acid, has emphasized his nuclear origin of 'nuclear protein→melanin protein'.

We also recognize the importance of the finding that the appearance of melanin granules in the form of nuclear caps at the distal end of the nuclei in the cells with enlarged nucleoli in the upper layer of the Malpighian rete. We cannot agree in particular with the opinion that this finding is merely due to the transition of melanin from the basal layer or the elongation of the processes of the dendritic cells (Becker and Fitzpatrick[4]). We are rather inclined to think that the mobility of the nucleic acid involves the promotion of metabolism of nuclear protein and the consequent activation of oxidizing enzymes, expediting the oxidizing reaction for melanogenesis in the cells in the upper layer. Simultaneously with the present ultraviolet irradiation experiments we carried out tests for peroxidase by Sato-Sekiya's cupric sulfate method and Okano's C method and gained weakly positive results that there were peroxidase granules in the cytoplasm in the cells of the upper layers of the Malpighian rete, but suffice it to state that the reactions were too unstable to bring decisive results.

Summary

An ultraviolet irradiation was performed on normal human skin and its influences were histochemically investigated. An increase of RNA and the appearance of melanin granules in the form of nuclear caps in the upper layer of the Malpighian rete led us to presume that melanin might be produced in cells different from 'melanocytes' named by American dermatologists.

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III. Histochemical Investigations of Unna's Oxygen and Reduction Areas by Means of Ultraviolet Irradiation

The significance of dermo-epidermal pH in melanogenesis has been discussed by Dejust[1], Ropshaw, Caillau[2] and others. Considering that melanogenesis is an oxidation process by enzymes, this problem has a proper reason to be investigated. Lerner, Fitzpatrick *et al.*[3] reported that the induction period in tyrosine-tyrosinase reaction is infinitely prolonged at pH of above 8.5 or below 5.0, and Yoshida and Togashi[4] investigated the same point by their histochemical methods. We[5],[6] studied this problem from another point of view, using Unna's oxygen-reduction staining. And the results of Unna's method applied to the skin of patients with nevus pigmentosus, nevus fusco-caeruleus Ota, Riehl's melanosis, malignant melanoma, vitiligo and normal persons, and those observations by means of hydroquinone applications were reported in the previous monograph and the significance of biochemical changes of oxygen- and reduction-areas (Unna) in melanin formation was emphasized. In the present experiments, we have applied this Unna's method on the normal skin of the thigh, axilla and palm irradiated with ultraviolet rays.

Experiments and Results

Unna's method applied in the present experiments as follows: The oxygen area (Sauerstofforte) and the reduction area (Reduktionsorte) are examined by means of permanganate methylgreen method upon frozen sections. The oxygen area was stained with methylgreen and the reduction area with potassium permanganate.

1) Results in the skin of the thigh and axilla: In the skin slices excised one hour after 20 minutes' irradiation, we have found that the Malpighian rete at the irradiated site is somewhat changed to oxygen area as compared with that at the adjacent non-irradiated site (Fig. 5), and in the skin slices excised 3, 6 and 24 hours after irradiation, this difference can not be found.

2) Results in the skin of the palm: In the skin of the palm, is seen a different change of oxygen and reduction areas from that in the skin of the thigh and the axilla. Normal skin slices from the palm show a methylgreen stained oxygen area spreading all over the Malpignian layer up to immediately beneath the granular layer (Fig. 6), making a contrast to the reaction behavior of the skin from the thigh and axilla. However,

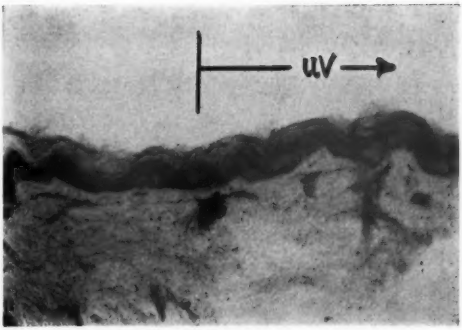

Fig. 5. Normal skin of the thigh (Unna's O-R-stain). uv→ultraviolet-irradiated site.

Fig. 6. Normal unirradiated skin of the palm (Unna's O-R-stain).

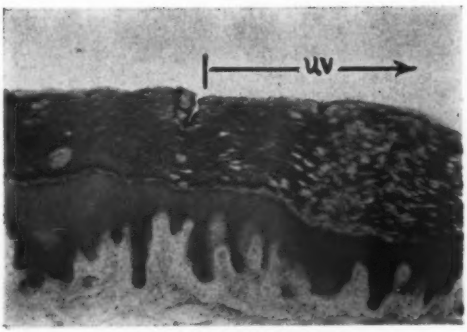

Fig. 7. Normal skin of the palm (Unna's O-R-stain). uv→ultraviolet-irradiated site.

the skin slices excised from the palm one hour after 20 minutes' ultraviolet irradiation show a reduction area stained brown by potassium permanganate spreading down to the lowest prickle-cell layer (Fig. 7), presenting, as it were, a process of the oxygen content in the Malpighian cells having been consumed by irradiation and making a similar appearance to what is observed in non-irradiated skin slices from other portions of the body. Moreover, Unna's method applied to a pigmented nevus excised from the palm, and to a pigmented spot from the neck where the melanin pigmentation reappeared of a patient with complete vitiligo has revealed that in the site containing melanin in the basal layer the layer above it shows a reduction area, while in the adjacent sites the oxygen area is spreading up to the upper Malpighian layer, quite in accord with the physiological findings of the palm.

Comment

From the results as mentioned above, it has made clear that the skin with melanin formation such as thigh, axilla, the palm with nevus pigmentosus, and the repigmented neck of complete vitiligo shows a phase difference, that is, the basal layer is stained as an oxygen area and the prickle-cell layer as a reduction area, and that the skin of the nonpigmented site such as palm shows no phase difference. This seems to give a suggestion to the demonstration of the local characteristics of the absence of melanin in the palm and the sole.

Urbach and Kral[7] demonstrated that a pigmentation by an application of bergamot oil followed by ultraviolet irradiation, was inhibited by the injection of ascorbic acid or riboflavin. In the previous monograph, we[6] confirmed the same fact and noticed that reduction area increased both in the epidermis and in the dermis of the skin in which pigmentation was inhibited by the injection of ascorbic acid. This coincides with the above-stated findings in the normal epidermis from the palm where no melanin formation occurs. In other words, the phase difference as a condition of the field of melanin formation was not demonstrated in the case associated with ascorbic acid injection. Thus we are led to emphasize the importance of Unna's oxygen and reduction areas which open the problem of ' field ' in considering the positive and negative enzyme reactions in the colored and colorless portions of the skin. Consequently we are of the opinion that the presence of melanin-free dendritic cells in the basal layer of the palm skin, as pointed out in our above-described iron-alum hematoxylin staining, and the presence of the dendritic cells (Langerhans' cells) demonstrated likewise in the Malpighian rete being unable to produce melanin can be explained from the angle of phase difference of oxygen- and reduction areas in the field of

melanin formation.

SUMMARY

Oxygen and reduction areas (Unna) were examined before and after an ultraviolet irradiation in specimens from the normal skin of the thigh, the axilla and the plam, and then from a pigmented site of the palm with nevus pigmentosus and a repigmented site of the neck of a patient with complete vitiligo. The skin with melanin formation shows a phase difference, that is, the basal layer is stained as an oxygen area and the prickle-cell layer as a reduction area. On the contrary, the skin of nonpigmented site such as the palm shows no phase difference. Ultraviolet irradiation can bring on a temporary change of this condition on the palm. From these results it was emphasized that the phase difference is very important as a condition of the field of melanin formation, and that the dendritic cells in the basal layer of the palm can not produce melanin because of no phase difference in the epidermis.

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IV. Mast Cells and Melanin Formation

The correlation of pigment cells and mast cells in the dermal layer has, since as early as the late 19th century, been noticed by Weidenreich, Bogoliabski, Koebner, Karg, Philippson, Werman and Rheindorf. Staffel[1] has inferred an affinity of the two from their arrangement around the vascular walls and a similarity in their spindle-like and dendritic shapes, and Meirowsky has pointed out the similarity in size of the granules in melanoblasts and mast cells in the dermal layer. Lipschütz[2] smeared Wien gray mice with tar and found in them an increase in number of mast cells accompanying the appearance of elongated, fibroblast-like pigment cells arranged parallel with the skin surface in the corium, which he took to be melanin cells because they were discolored by H_2O_2. Besides, infiltration of mast cells has long been known to constitute the principal finding in urticaria pigmentosa, and it is not difficult to suppose from this and clinical findings of pigmentation that there is a correlation between this mast cell infiltration and the increase of melanin in the epidermal basal and dermal papillary layers. More recently, it has come into general acceptance that mast cells produce hyaluronic acid (Asboe-Hansen[3]) and heparin, both mucopolysaccharides, and take part in histamine secretion. Schuppli[4] has found that heparin expedited the formation of melanin from dopa in an in vitro experiment, and that the intracutaneous injection of heparin expedites melanogenesis in rabbits. Igarashi, Saito and Aizawa[5] of our clinic have succeeded in inhibiting eruptions and finally extinguishing the pigmentation in urticaria pigmentosa by repeated injections of hyaluronidase.

Experiments and Results

1) We have examined histological preparations from Mongolian spot, blue nevus, nevus fusco-caeruleus (Ota and Itô) and nevus pigmentosus (fascicular type), by Ohno's method (a modified Lison method).

The mast cells and pigment cells are nearly identical both in form and in size, and they are placed so close together (Figs. 8, 9) that in some cells mast cell granules and melanin granules are seen to coexist in the same cytoplasm (Fig. 10).

2) The embryological correlation between these two kinds of cells has also been investigated by Ohno's method with skin samples from the sacral region in 12 fetuses (1 of 8th week, 1 of 9th week, 2 of 11th week, 2 of 12th week, 1 of 14th week, 3 of 5th month, 1 of 6th month and 1 of

8th month) and 8 newborns.

The mast cells and Mongolian spot cells first appear close together in the 14th week. In their early stages, it is imposible to distinguish them by their forms or sizes, only possible by their colors. The mast cells are more widely distributed in the skin and have for a longer time been present in the subpapillary layer than the Mongolian spot cells.

Comment

Mast cells as well as cell nuclei in general cells have been considered by Unna[6] to be principal oxygen reservoirs, pointed out as Unna's primary sites of oxygen (primäre Sauerstofforte) because they have peroxidase, and supposed to play a part in the oxygen supplying process from the blood and lymph to fibrous connective tissues. Hence it is considered, on the analogy of the mechanism of melanogenesis in the epidermal basal cells, in which oxidase and peroxidase participate as pointed out in our previously reported histochemical examinations[7], that mast cells participate as a factor in the stimulation of oxidation for melanin formation in the cells of the Mongolian spot, blue nevus and nevus fusco-caeruleus present in the connective tissues of the corium.

Summary

The correlation of dermal melanin forming cells and mast cells was studied. Mast cells were presumed to take a role as a primary oxygen area (Unna) in melanogenesis in melanin forming cells of Mongolian spot, blue nevus and nevus fusco-caeruleus.

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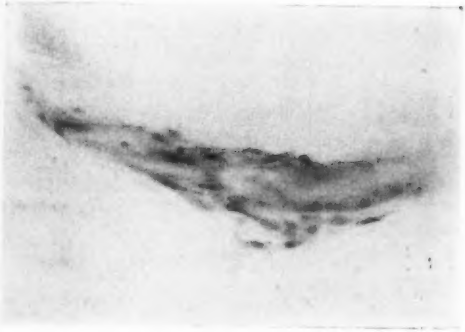

Fig. 8. Mongolian spot (Lison's method modified by Ohno).

Fig. 9. Pigmented nevus of fascicular type (Same stain).

Fig. 10. Nevus fusco-caeruleus acromio-deltoideus (Same stain).

V. Tyrosinase Reaction

For histochemical demonstration of tyrosinase, there is the ultraviolet ray irradiation method devised by Fitzpatrick *et al.*[1),2)]. The present authors have devised methods using $CuSO_4$, part of which has been published previously[3)]. In this report, we will discuss some fundamental conditions for effective application of our method, and the influence of some substances on its results. For easier understanding of our discussion, we will first give rough outlines of Fitzpatrick's method and ours.

The Method of Fitzpatrick et al.

1) Ultraviolet irradiation for 5 to 8 consecutive days with erythema doses.

2) The reddened part is excised and 1 to 3 mm. slices of tissue are fixed in 10% neutral formalin for 1 hour.

3) The slices are incubated in 0.005-molar L-tyrosine in 0.1-molar phosphate buffer at pH 6.8 in the cold (0° to 4°C) for 24 hours.

4) They are kept in tyrosine at 37°C for 24 hours.

5) Further fixation is carried out in formalin for 24 hours and then sections are made.

The Methods of Yoshida and Togashi

First Method

1) 1% of cupric sulfate solution is intracutaneously injected into the unirradiated skin.

2) The injected part is removed after 2 hours and fixed in 10% neutral formalin for 1 hour.

3), 4) and 5) Same as in Fitzpatrick's method above.

Second Method

1) Unirradiated skin is removed.

2) 0.125–1.0% cupric sulfate solution is intracutaneously injected into the removed skin and fixation in 10% neutral formalin for 1 hour at 0°– 4°C.

3), 4) and 5) Same as above (Figs. 11, 12).

Third Method

1) Unirradiated skin is removed.

2) Incubation in 0.25–0.5% cupric sulfate solutions for $\frac{1}{2}$–1 hour at 0°– 4°C. Fixation for 1 hour in 10% neutral formalin.

3), 4) and 5) Same as above (Figs. 13, 14).

Variation of Methods I–III.

The process 3) above may be omitted.

Fourth Method

Approximately 24 hours after irradiation with ultra-violet rays for a short duration (say, for 10 minutes, once, an irradiation too weak to activate the tyrosinase by Fitzpatrick's method) is applied our Second or Third Method. A stronger positive reaction can be obtained by this combined method than by any of the above methods singly applied.

Studies on the Various Conditions

1) pH: Any pH value of the phosphate buffer solution within the range of 6.4–7.4 does not inactivitate the tyrosinase activity, but when a value exceeds 8.0 or falls below 6.2, the activity is inactivated.

2) Fixation: When the time of fixation in 10% neutral formalin is more than 3 hours, the activity is weakened and if it is over 4 hours, the activity is inactivated. When alcohol is used for fixing, one hour is enough to make the activity inactive.

3) The activity is stable, if tissues are kept in 0.9% NaCl solution at 0°C, even for 6 days after excision.

4) When tissues are placed in 0.25% $CuSO_4$ and kept at 4°C for 3 hours, the activity is weakened and will in 15 hours be entirely inactivated.

5) Boiling for 5 minutes at 100°C will inactivate the activity.

6) Variation by the skin sites: Even our methods, if followed exactly, will not always cause positive reaction. For example, under our First Method, 6 out of the 7 sections from the skin of the axilla showed positive and 1 negative results. Under our Second, Third and Fourth Methods, 8 of the 86 sections from the axilla skin reacted negatively, while 1 of the 4 sections from the back skin showed also negative reaction. Evaluation was often difficult, when the tissues tested were from the neck, the chest, the back of the shoulder, the upper or the forearm, the groin and the thigh. When the skin of the right and the left axilla was tested on different occasions, the same individuals showed positive results on both sides in 10 instances, negative on both sides in 2, and showed different reactions between the two sides in 2.

7) When the skin tissues were placed in 100 cc of M/15 phosphate buffer (pH 6.8) to which 100 mg of tyrosine and 2 mg of $CuSO_4$ had been added and kept at 0°– 4°C for 1 hour and then incubated for 24 hours at 37°C therein, 3 of the 10 tested tissues showed positive results.

Investigation of Some Tyrosinase Activation Methods Using Various Compounds Other than $CuSO_4$

1) CuCl and $CuCl_2$: Using these compounds in varying concentrations instead of $CuSO_4$ in our Fourth Method (CuCl is soluble in 1000 cc

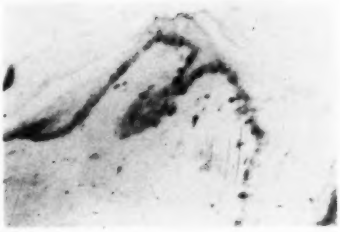

Fig. 11. Second method. (× 320)

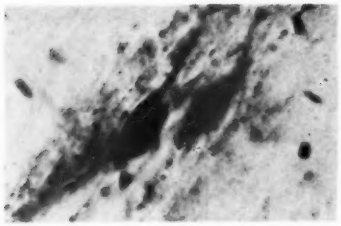

Fig. 12. Second method. (× 1200)

Fig. 13. Third method. (× 320)

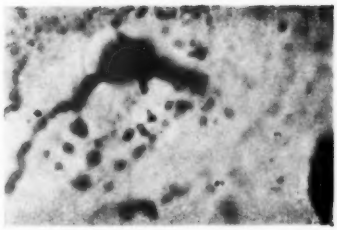

Fig. 14. Third method. (× 1200)

of water at 0°C only to the extent of 0.0062 g and at 25°C of 1.53 g, and the solute turns into $CuCl_2$ almost completely), we found that a rather stronger positive reaction can be obtained with a saturated solution of CuCl than with $CuSO_4$, but when the concentrations were lowered to 1/100–1/100,000 of the saturated solution or 1–0.000001 M $CuCl_2$ solutions were used, the results were always negative.

2) Iodacetamide: This was tried likewise in various concentrations. By incubation in 0.001–0.000001 M solutions for 30–60 minutes, only 1 specimen immersed in 0.001 M solution for 30 minutes was found to show positive reaction, among the 21 total specimens tested variously.

3) Estrogen: In applying our First Method, we used here a 0.2 mg/1 cc suspension or a 5 mg/1 cc water solution of estrogen in place of $CuSO_4$. Four of the 12 tested specimens showed positive results, but all the 8 by *in vitro* tests, following our Second, Third and Fourth Methods, showed negative results.

Activation of Tyrosinase by Single Long Irradiation with Ultraviolet Rays

The right thigh of a man, 27, was irradiated on its extensor side from a distance of 30 cm for 20, 40 and 60 minutes, and the skin was resected 1, 3, 5 and 7 days thereafter. The results of tyrosine reaction following Fitzpatrick's method in principle are shown in Table II.

TABLE II

Activation of Tyrosinase by Single Long Irradiation with Ultraviolet Rays

Irradiated for (minutes)	Removed (days) after irradiation			
	1	3	5	7
20	−	−	−	−
40	−	±	+	−
60	−	−	±	−

Influence of Various Substances on Tyrosinase Reaction in Our Methods

Skin sections obtained from the axilla 2 to 3 mm in size, were kept in a 0.25% $CuSO_4$ solution for 1 hour at 40°C, rinsed with water and incubated for 1 hour in solutions of the test compounds at 4°C, rinsed again with water, then incubated for 24 hours at 37°C in 10 cc of 1/15 M phosphate buffer (pH 6.8), to which 10 mg of tyrosine had been added. Then the tissues were fixed in 10% neutral formalin and made into slide sections.

The results of the tests were entirely negative in the cases of the following compounds: 70% alcohol, aceton, aniline, BAL, 1/10 M cystein hydrochloride, " Strong Interenin " (adrenal preparation), ether, 1/12 M ferrous chloride, 1/12 M glucuronic acid, 1/40 M guanofuracin, 1/8 M L-methionine, 1/8 M thioglucollic acid, 1/10 M potassium cyanide, 1/20 M pyruvic acid, sodium heparin (1000 International Units per 1 cc), 1/3 M sodium thiosulfate, 1% sulfuric acid, 3 M glysine, 10% thiasin, 1/7 M thiouracil, 1/5 M thiourea, 1/5 M urea.

Relationship between the Concentration of Reaction-inhibitive Compounds and Their Effects on Tyrosinase Reaction

The skin tissues taken from the axilla, 2 to 3 mm in size, were incubated in solutions of the tested compounds for 1–2 hours, rinsed with water, incubated in a 0.01 M $CuSO_4$ solution for 1–2 hours at 0°–4°C, again water-rinsed, incubated in 10 cc of phosphate buffer solution (pH 6.8) to which 10 mg of tyrosine had been added, fixed in 10% neutral formalin and made in to slide preparations (The pre-Cu-activation method).

2) The tissues were first incubated in a $CuSO_4$ solution prior to the treatment with the solution of test compound, as in the method above (The post-Cu-activation method).

3) The tissues are incubated in a mixture of 0.5% $CuSO_4$ and the solution of test compounds (The simultaneous method).

The results of tests under these methods in collation with the control tests conducted under the regular method, are shown in Table III, where (+) stands for a specimen showing the same results as in the control tests,

TABLE III

Influence of Various Compounds on Tyrosine-Tyrosinase Reaction. (Values show M concentrations)

Methods	pre-*			post-*			simul-*		
Reaction-results	−	±	+	−	±	+	−	±	+
Thioglycollic acid	$1\sim10^{-2}$		$10^{-3}\sim10^{-5}$	$1\sim10^{-2}$		$10^{-3}\sim10^{-5}$	$1\sim10^{-2}$		$10^{-3}\sim10^{-5}$
L-Methionine		1	$10^{-1}\sim10^{-5}$		1	$10^{-1}\sim10^{-5}$	1	10^{-1}	$10^{-2}\sim10^{-5}$
Sodium thiosulfate	$1\sim10^{-1}$	10^{-2}	$10^{-3}\sim10^{-5}$	$1\sim10^{-1}$	10^{-2}	$10^{-3}\sim10^{-5}$	$1\sim10^{-2}$	10^{-2}	$10^{-3}\sim10^{-5}$
Methylthiouracil	$1\sim10^{-1}$		$10^{-2}\sim10^{-5}$	$1\sim10^{-1}$		$10^{-2}\sim10^{-5}$	$1\sim10^{-1}$	10^{-2}	$10^{-3}\sim10^{-5}$
Thiourea	1	$10^{-1}\sim10^{-2}$	$10^{-3}\sim10^{-5}$	1	$10^{-1}\sim10^{-2}$	$10^{-3}\sim10^{-5}$			$1\sim10^{-5}$

pre-*, post-*, or simul-* shows pre-, post-, or simul-Cu-activation method respectively.

(±) showing a weakened and (−) a negative result.

Comment

The idea that tyrosine is the mother substance in melanin formation and tysinase has the highest importance as the oxidase in it has long been accepted. Since, however, Bloch discovered the dopa reaction, the theory that normal melanogen in man is dopa and the catalyst responsible for conversion of dopa to melanin is dopa oxidase has come to receive strong support. The experiments by Hogeboom and Addams[4] (1942) on Harding-Passey mouse melanoma, the follow-up studies thereof by Greenstein[5] and the detailed researches by Lerner *et al.*[6),7] succeeded in demonstrating the presence of tyrosinase activity, at least in extracts of malignant tissues. On the other hand, repeated attempts have been made to show tyrosinase activity in normal tissues; but definite results have not yet been obtained by biochemical methods. Fitzpatrick *et al.*[1),2], however, tried to demonstrate tyrosinase in normal human skin by histochemical method, and finally succeeded in the attempt by means of the ultraviolet irradiation method as stated above. They concluded: (1) The tyrosinase reaction appears to be associated with the malignant character of human melanocytes; (2) non-malignant character of human melanocytes in normal skin requires activation of the tyrosinase system by a stimulating factor (for example, radiant energy) in order to form melanin when incubated in tyrosine. They also emphasized the high significance of tyrosinase reaction in diagnosing malignant melanoma. They admitted, however, that the mechanism by which ultraviolet radiant energy activates the human tyrosinase is yet unknown; they had failed in the attempt to prove the following two possibilities: (1) Dopa formed by direct photochemical oxidation of tyrosine present in the tissues may catalyze the enzymatic oxidation of tyrosine to melanin, and (2) ultraviolet radiant energy may decrease the concentration of normally occurring sulfhydryl groups in the epidermis. We have made follow-up experiments with their histochemical method and obtained results similar to those reported by them. But the results were not always constant, and besides it was obviously difficult to administer to human skin an erythema dose daily of ultraviolet light for 7 days in order to study this method from various angles with ample materials. Ultraviolet irradiation needed to be replaced by some more practical device. Our experiments using numerous compounds in search for such a device succeeded at length in developing the above-described method with $CuSO_4$[3]. By this method of ours, it has for the first time become possible to experiment without causing undue inconvenience to the subject. Of various metals, copper has long been known to have a close relation to melanin formation; it is also known to be an

active radical of tyrosinase, whether in plant or animal, and to catalyze the oxidation of -SH groups which are known to be inhibitory factors in melanin formation. Clinical observations also have suggested the important significance of copper in pigmentary anomalies. But these facts do not mean that the role played by copper in the process of melanin formation has been established; many questions remain unsolved. In our method, the mechanism by which $CuSO_4$ activates human tyrosinase is yet to be elucidated by future researches.

Biochemical studies[1] have already established that with mouse melanoma tyrosinase the optimal pH for oxidation of dopa is about 6.8, and that the induction period is prolonged indefinitely when the pH value is above 8.5 or below 5.0. There is, however, no histochemical study reported on the influence of pH on tyrosine-tyrosinase reaction. We have proved that, under the above conditions or at a pH ranging from 6.4 to 7.4, the reaction was positive.

In respect of the effects of various fixatives, we have found that fixation in a 10% neutral formalin solution for more than 4 hours gives a negative tyrosine-tyrosinase reaction. This shows that the reaction is easier to be inhibited by formalin fixation than dopa reaction. Alcohol fixation for only one hour makes the reaction negative. These results are in agreement with the remarks by Fitzpatrick[1] that fixation of the tissue with alcohol or formalin inactivates tyrosinase activity. Our observation that, when the tissue is kept in a 0.9% NaCl solution at a low temperature (0°–4°C), the reaction is positive even at 6 days after resection of the tissue is also in accord with Fitzpatrick's statement that tyrosinase is stable for 1–6 months when tissues are kept in a deep freeze.

Even our method could not always bring about positive reactions; the fact that by this method the positivity rate was highest with the skin tissues from axillae and relatively low with those from other regions of the body seems to indicate that the conditions most favorable for testing the skin tissues from axillae are not always the optima for tissues from other sites, and that the optimal conditions are not always the same with different human individuals. The determinations of reactions with the skin tissues from both axillae of one and the same individual at different occasions show that the results are not unreliable at all times. At present, however, the mechanism of our method being yet unclarified, it is impossible to explain fully the difference in reaction according to different regions from which the skin tissues have been excised. Further studies are required for the method of treating tissues with a mixed solution of $CuSO_4$ and tyrosine.

In the results obtained by the activating methods with compounds other than $CuSO_4$, we may point out that the difference between CuCl

and $CuCl_2$ might have some relation to the difference between cuprous and cupric ions in these compounds. Whether the fact that estrogen, while activating tyrosinase in vivo, failed to show the same effect in vitro was due to an action mechanism of estrogen different from that of Cu or to the possible inadequacy of our in vitro method is a question which cannot be answered hastily. The tyrosinase reaction induced by one prolonged ultraviolet irradiation has already been discussed in connection with the results of other histochemical tests.

Studies on inhibitors of melanin formation have been made in vitro mainly by Warburg's manometric method and on the basis of clinical observations, but no reports have been published on their effects on tyrosinase reaction viewed from the histochemical angle. Our method makes it very easy to perform such studies. A caution, however, is needed in evaluating the results, whichever one of our pre-, simul- and post-Cu-activation methods is adopted. For in case a reaction is turned negative by addition of a test substance, we should judge whether this effect is due to the inhibitory action of the substance on the activation of tyrosinase by Cu, or on the reaction of activated tyrosinase on tyrosine, i.e. the tyrosine-tyrosinase reaction. In the case, however, of a positive reaction, we may say as much as that the compound has no influence on the reaction in a given concentration under a given test condition. Table III examined with the above fact in mind will show that the substances which are considered to inhibit melanin formation by combining with copper allow positive reactions in certain concentrations: thioglycollic acid in a concentration below 1/1000 M (pre-, post-, and simul-Cu-activation methods); L-methionine, below 1/10 M (pre-, post-) or 1/100 M (simul-); sodium thiosulfate, below 1/1000 M (pre-, post-, and simul-); methylthiouracil, below 1/100 M (pre-, post-), 1/1000 M (simul-); thiourea 1/1000 M (pre-, post-), 1 M (simul-).

The results of ascorbic acid, monobenzylether of hydroquinone and guanofuracin were very complicated. The details on this point will be described in the near future.

Summary

1. A new histochemical method of activating tyrosinase using $CuSO_4$ is presented by the authors, and discussions have been conducted on experimental conditions, such as the pH value, the fixative, the time of fixation, and the sites of sampling.

2. Activation methods using compounds other than $CuSO_4$ were also examined. The possibility of obtaining similar activation by use of saturated CuCl solution was demonstrated, and it was established that intracutaneous injection of estrogen in some cases may have the same effect.

3. Our method has made possible a histochemical examination of the influence of various compounds on tyrosine-tyrosinase reaction. Experiments were made with some compounds considered to inhibit melanin formation by combining with copper.

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VI. Serum Tyrosine and Melanin Formation

On Seasonal Fluctuation of Serum Tryosine

It was reported by Jesionek and Rothman[1] that the serum tyrosine content is higher with the black race than with the white, and in individuals who are subject to skin pigmentation by exposure to sun-light than in those who are not. It was confirmed by Steiger-Kazel's[2] follow-up tests, and about the same results were reported by Matsumoto[3] who, finding that the blood tyrosine content falls lower in summer when the sun is stronger and the daytime is longer than in winter, supposed, in accordance with Rothman's view, that this is because tyrosine is consumed in larger quantities in summer, probably by increased production of melanin. And Fujino[4] recognized in guinea-pigs irradiated with ultraviolet rays that the tyrosine content in the serum decreases and that in the skin extract increases after the irradiation.

We determined the serum tyrosine contents of 920 patients with various skin diseases by the nitrosonaphthol method as described by Udenfried and Cooper[5], and classified the results by the months in which the blood was taken. Though individual values showed some variation, the mean value showed a tendency to rise in winter and to fall in summer, and the descending curve in early summer was comparatively rapid whereas the ascending curve in autumn was slow (Table IV & Fig. 15). Further examinations of patients with various pigmentary diseases, namely, nevus pigmentosus (68 cases), nevus fusco-caeruleus Ota (53 cases), ephelides (86 cases), chloasma (110 cases), Riehl's melanosis (31 cases), postinflammatory pigmentation (37 cases), vitiligo (93 cases), and albinism (18 cases), and 94 normal persons as controls showed that the results classified by the months of blood collection described about the same curve as in the above statistics of all patients with general skin diseases.

Repeated quantitative experiments over a period of one year to evaluate serum tyrosine in 6 normal persons, 2 patients with chloasma, 2 with Addison's disease, 1 with lichen pigmentosus, 1 with albinism, and 1 with vitiligo showed that the value is lower in summer, and higher in winter, in one and the same individual (Table V).

The exact values of serum tyrosine varies more or less with the month, but for convenience' sake, the average for November through April (the winter value) and that for May through October (the summer value) are used in our comparison of the difference between the two (Table VI). Here the difference is about the same extent in patients with nevus

TABLE IV

Monthly Values of Serum Tyrosine Content in 920 Cases

Month \ Serum tyrosine mg/dl	0.5–1.0	1.01–1.5	1.51–2.0	2.01–2.5	2.51–3.0	3.01–3.5	3.51–4.0	4.01–	Total Number of Cases
Jan.		1	7	14	13	10	2	2	49
Feb.			11	19	19	7	4	2	62
Mar.		2	20	22	18	6	6	2	76
Apr.		2	17	16	23	12	3	2	75
May	4	45	31	6		1		1	88
June	21	92	22	2					137
July	24	66	9	3					102
Aug.	22	48	13						83
Sept.	4	33	22	3					62
Oct.	1	24	38	19	5	2			89
Nov.		1	18	13	9	6	3	1	51
Dec.		4	12	7	13	5	3	2	46

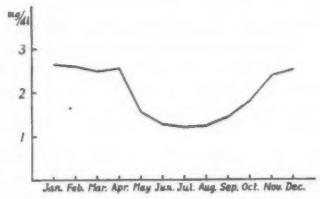

Fig. 15.

pigmentosus, nevus fusco-caeruleus or ephelides as in normal persons, while it is somewhat smaller in patients with chloasma or Riehl's melanosis, and much greater in those with postinflammatory pigmentation, Addison's disease, vitiligo or albinism, than in normal persons. The difference between both season values of serum tyrosine in individuals, is somewhat smaller in patients with chloasma, and much greater in those with Addison's disease, lichen pigmentosus, vitiligo or complete albinism than in normal persons. Thus, the differences in individuals (Table VII) present a similar tendency as in above-stated mean values (Table VI).

Discussion

The above results of ours show a similar tendency to the observations by Rothman and Matsumoto. The seasonal fluctuation of the values

TABLE V

Monthly Values of Serum Tyrosine in Individuals

	Age	Sex	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Normal control	27	F	2.12	2.79	1.92		1.81	1.40		1.84	1.59	2.08	2.38	2.56
"	29	M	1.76	2.54				1.15	1.34	1.56		1.99		1.99
"	34	M	2.94	2.75		2.86		1.44	1.65	1.57		2.06	2.61	3.21
"	32	M			2.21				1.79		1.52	1.89		3.02
"	37	M	3.38	3.27		4.20				1.30		1.81	3.53	
"	26	M		3.82	2.58	1.62		1.14	1.31	1.54	1.58	2.19	3.38	3.73
Chloasma	25	F	1.74	1.86	2.00		1.70	1.45	1.36	1.15	1.22	1.93	2.40	
"	23	F	2.61	2.97	1.86		1.39	1.42		1.84	1.55	2.40	2.40	
Addison's disease	29	M	3.12	2.86	3.32		1.50		1.50	1.07	1.67	1.78	2.07	2.59
"	29	M		2.64		3.41		0.94		1.40	1.42	1.76	2.68	3.07
Lichen pigmentosus	38	M	3.38				1.45		1.52			1.57		2.71
Albinism	11	F	2.99		2.90	3.65	2.24		1.99		2.39	2.85	3.38	3.97
Vitiligo	20	F	3.36	3.74	2.56	2.95	1.59					1.42	1.75	3.30

TABLE VI

Difference in Mean Value of Serum Tyrosine between Summer and Winter

	No. of determ.	Winter values mg/dl	(cases)	Summer values mg/dl	(cases)	Difference mg/dl
Normal controls	94	2.62	(35)	1.56	(59)	1.06
Nevus pigmentosus	68	2.34	(30)	1.27	(38)	1.07
Nevus Ota	53	2.50	(29)	1.36	(24)	1.14
Ephelides	86	2.32	(42)	1.30	(44)	1.02
Chloasma	110	2.30	(49)	1.31	(61)	0.99
Riehl's melanosis	31	2.25	(15)	1.33	(16)	0.92
Postinflammatory pigmentation	37	2.81	(13)	1.31	(24)	1.50
Addison's disease	35	2.83	(15)	1.54	(20)	1.29
Vitiligo	93	2.68	(31)	1.39	(62)	1.29
Albinism	18	3.09	(8)	1.77	(10)	1.32

seems to reflect the behavior of serum tyrosine consumed according to the change in the length of sunshine and the quantity of ultraviolet rays. Besides, the rapid fall of the curve in early summer may suggest that melanin is actively produced for protecting the skin from the action of the sunlight in that season, and the gentle curve of increase in autumn can be interpreted as indicating the gradual decrease in melanin formation in the season.

In the studies by disease types, the fact that the nevus pigmentosus, nevus fusco-caeruleus, chloasma and Riehl's melanosis cases show meanly the same difference in mean value between summer and winter as normal persons seems to indicate that these diseases are localized melanosis showing little seasonal fluctuation of pigmentation, so that they normally consume a nearly constant quantity of melanin within the range of the physiological fluctuation of normal persons. On the other hand, the greater difference shown by vitiligo cases suggests that they are very susceptible to the action of sunlight and it may be considered in collation with the clinically observed frequency of hyperpigmented haloes around vitiligo foci. The high value shown by albinism cases both in summer and winter is probably due to the fact that the pathogenesis of this disease is based on a prenatal deficiency in oxidase which is needed in the process of melanogenesis and thus it is not directly correlated with the metabolism of tyrosine which is the precursor of melanin. The large difference between the summer and the winter values shown by such cases suggests that these patients are oversensitive to ultraviolet rays, that is, the metabolism of the liver etc. thereby receives a strong stress.

The small difference between the summer and the winter values shown by the ephelides cases who show intensified pigmentation in summer is somewhat against what we have anticipated, but in such cases, in consideration of the results obtained in the experiments described in a following chapter, with added ascorbic acid and the instability of content of ascorbic acid in, their plasma there seem to be some factors complicating the matter.

In the case of Addison's disease, the pathogenesis is due to a hypofunction of the adrenal glands, and many have been the reports on the complex relations concerning adrenalin and tyrosine, so that we cannot give off hand conclusions on the fluctuation of tyrosine values determined by us.

Fluctuation of Serum Tyrosine following Sun-bathing

As the above fluctuation in the seasonal values of serum tyrosine presumably reflects the effect of sun-light over a long period, we next studied the effects of transient but intense exposure to ultraviolet rays, with 16 normal Japanese who were asked to take sea- and sun bathing on one or two days in mid-summer, making measurements of their serum tyrosine values before and after bathing. The results are shown in Tables VIII and IX. The serum tyrosine value one week after bathing as compared with the pre-bathing value showed a greater increase in subjects who were exposed stark-naked to greater amounts of direct sunlight, and darker complexioned Japanese showed a higher and more persistent rise in tyrosine value than the fair complexioned Japanese, both 1 and 3 weeks

TABLE VII

Difference in Serum Tyrosine of Individuals between Summer and Winter

	Age	Sex	Winter values mg/dl	Summer values mg/dl	Difference mg/dl
Normal control	37	M	4.20	1.30	2.90
„	34	M	3.20	1.44	1.76
„	26	F	2.79	1.27	1.52
„	32	M	3.02	1.52	1.50
„	29	M	2.59	1.15	1.44
„	28	M	2.54	1.36	1.18
Nevus pigmentosus	25	F	2.65	1.22	1.43
Nevus Ota	20	F	4.64	0.88	3.76
„	21	F	2.94	1.14	1.78
„	23	F	2.40	1.31	1.09
Chloasma	23	F	2.87	1.12	1.75
„	23	F	2.97	1.55	1.42
„	24	F	2.55	1.15	1.40
„	39	F	2.45	1.17	1.28
„	25	F	2.20	1.06	1.14
„	24	F	2.09	1.15	0.94
„	24	F	2.04	1.17	0.87
Riehl's melanosis	36	F	3.01	0.89	2.12
„	36	F	1.71	1.20	0.51
Addison's disease	29	M	3.41	0.94	2.47
„	29	M	3.32	1.07	2.25
„	40	M	3.34	1.85	1.49
„	34	M	2.75	1.30	1.45
Lichen pigmentosus	38	M	3.38	1.45	1.93
Melanoma	66	F	3.29	1.95	1.34
Vitiligo	20	F	3.74	1.41	2.33
„	19	M	3.04	0.87	2.16
„	51	F	2.72	1.22	1.50
Albinism	20	F	3.11	0.94	2.17
„	11	F	3.94	1.99	1.98

after bathing.

Large areas of the back of a rather dark complexioned man (35 years) and a female case of albinism (20 years) were exposed to artificial sunlight at a distance of 50 cm for 30 minutes. The tyrosine values measured over a period 24 hours before and after irradiation are shown in Fig. 16. The tyrosine values rose 2 hours after irradiation in both cases, but while the man showed little change in the value for 9 hours thereafter the albino

TABLE VIII

Change in Serum Tyrosine Content of 1 Week after Sun-bathing

Age (years)	Sex	%
Persons with a bathing suit or seaside wear		
23	M	+12.5
27	M	+ 7.8
30	F	+ 7.3
28	F	+ 5.9
25	M	+ 4.9
23	F	+ 1.4
25	F	− 3.8
Average		+ 4.7
Persons with bathing drawers		
61	M	+50.0
27	M	+34.1
32	M	+33.6
27	M	+30.5
25	M	+21.1
28	M	+11.6
25	M	+ 8.5
29	M	+ 6.7
35	M	− 4.8
Average		+17.2

woman showed a decreasing tendency of tyrosine content. When the latter was exposed to infrared rays under the identical conditions, she showed hardly any fluctuation in her tyrosine value. Thus it was known that in experiments, the serum tyrosine content shows a higher increase rate in individuals with a greater and somewhat more persistent tendency to melanogenesis.

Discussion

Rothman stated that a dermatitis induced by intense action of light entails symptoms resembling those of dysfunction of the adrenal cortex and that the precursor substance of pigments increase in the blood, and Steiger-Kazal reported that those who are with fair skin and those who are prone to get sunburnt have more tyrosine in their serum than those with dark skin and those who are not easily sunburnt. Our experiments in irradiation with intense ultraviolet rays have reached about the same

TABLE IX

Change in Serum Tyrosine Content 1 and 4 Weeks after Sun-bathing

Age (years)	Sex	Luminosity %	After 1 week (July 29) %	After 4 weeks (Aug. 20) %
Dark complexioned				
61	M	38	+50.0	+57.3
32	M	25	+33.6	+33.6
27	M	35	+ 7.8	+20.3
29	M	35	+ 6.7	+16.4
23	F	36	+12.5	+10.7
average		34	+20.8	+26.7
Fair complexioned				
28	M	45	+11.0	+17.7
30	F	44	+ 7.3	+ 8.9
27	M	45	+34.1	+ 8.5
28	F	45	+ 5.9	+ 2.9
23	F	45	+ 1.4	+ 1.4
average		45	+12.4	+ 8.3

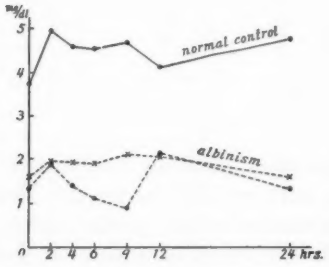

Fig. 16. • ultraviolet, × infrared.

results with these reports.

Fluctuation of Serum Tyrosine Following Administration of Ascorbic Acid

A comparison of the measurements of serum tyrosine in 91 patients with various pigmentary diseases and 3 normal subjects between before and 30 minutes after intravenous injection of 1,000 mg ascorbic acid is shown in Table X.

The serum tyrosine value showed little change in normal subjects

TABLE X

Change in Serum Tyrosine Content Following Injection of 1,000 mg Ascorbic Acid

	Cases	decreased		un-changed	increased	
		~10%	10~5%	±5%	5~10%	10%~
Normal controls	3			3		
Nevus pigmentosus	7		1	6		
Nevus Ota	7	3		3	1	
Ephelides	13	5	1	7		
Chloasma	20	6	1	10	2	1
Riehl's melanosis	5	1	1	1	1	
Addison's disease	5		4	1		
Lichen pigmentosus	2	1	1			
Postinflammatory pigmentation	6	3	2	1	1	
Leucomelanodermia (MBEH)	2	1		1	1	
Depigmentation postinfl.	2			1	1	
Vitiligo	14		2	8	8	1 (universalis)
Albinism	1					1

and in cases of nevus pigmentosus and nevus fusco-caeruleus, but in the cases of acquired hyperpigmentary diseases, a high frequency of decreased post-injection values was observed, while in some of the cases of hypopigmentation, a rise in serum tyrosine value was observed following the administration.

Discussion

Sealock[6] found that the products of tyrosine metabolism excreted in the urine of guinea-pigs fed with food poor in ascorbic acid disappear following its administration and demonstrated that ascorbic acid is in relation with the metabolism of L-tyrosine and L-phenylalanin. Keith and Rienitz[7] observed by Warburg's manometric method that the decomposition of tyrosine effected by a section of liver tissue of a scorbutic guineapig is far lower than that by a normal liver section, and that this tyrosine decomposing capacity is recovered by ascorbic acid administration. Similarly, Majima[8], Takagi[9] and Beppu[10] reported that the tyrosine metabolism is disturbed in ascorbic acid deficient guinea-pigs. Knox[11] described that an application of ascorbic acid to the liver of a rat causes enahanced decomposition of tyrosine. Sealock *et al.*[12] and Levin *et al.*[13] found that the products of tyrosine metabolism coming out in experimental alkaptonuria and in urine of premature newobrns disappear by administration of ascorbic acid. Kahler and La Crois[14] found that the excretion

of urinary melanogen after sun-bathing is inhibited by administration is ascorbic acid and were led to conclude that ascorbic acid suppresses the production of melanogen. Iwanaga[15] discovered a decrease of reducive ascorbic acid in the tissue of dysfunctioned liver. From these observations, it may be deduced that ascorbic acid is closely related with tyrosine metabolism in the liver. On the other hand, Morawitz[16] pointed out that the pigmentation in Addison's disease and a few other pigmentary diseases are alleviated by ascorbic acid administration. Since then, many reports on similar clinical successes have appeared. Ogoshi[17] have tried the ascorbic-acid-iontophoresis therapy on various hyperpigmentary diseases by a method similar to that of Schuppli, and obtained fairly good results with postinflammatory pigmentation cases and more or less perceptible decoloration in some cases of nevus fusco-caeruleus. Schade[18] has observed that the pigmentation caused by exposure to artificial sun-light is limited by administration of ascorbic acid.

In our experiments, if the results had shown reduction of serum tyrosine in all the cases following the administration of ascorbic acid, we might be allowed to conclude that it was due to the stimulated metablism of tyrosine in the liver; but since the fact was that some of the hypopigmented cases showed an increase of the value, it is clear that such an interpretation is inadequate here. However, Sealock[19] has discovered by Warburg's manometric method that in colored guinea-pigs the demand for ascorbic acid increased with rise in tyrosine metablism, whereas in albino guinea-pigs, the demand was not enhanced. This observation and our assumption that the administration of ascorbic acid inhibits melanin formation through reduction of quinone and thereby causes an increase of the mother substance tyrosine have led us to infer that in the case of hypopigmentation, the decomposition of the liver tyrosine is not much expedited by ascorbic acid, which is used in preference for inhibiting melanogenesis by reduction, so that the serum tyrosine content is increased, while in the case of hyperpigmentation, the decomposition of the liver tyrosine is expedited corresponding to the administration of ascorbic acid in demand, so that the serum tyrosine content comes to be reduced. When this is corraborated by the fact recognized in our examinations to be described below, that the ascorbic acid content in plasma is found diminished in the cases of Addison's disease, ephelides and chloasma, and we are here reminded also of the curative mechanism of ascorbic acid manifested in such hyperpigmentary diseases, we feel our so-far given explanation may be justified.

Itô and Nakajo[20] of our laboratory have observed in their experiments in intensifying and reducing the skin coloration of gold-fish that the addition of vitamins C and B_2 respectively expedited both oxidizing

and reducing actions, and found in their test of exposing human skin smeared with bergamot oil to artificial sunlight that the pigmentation due to such a treatment is effectively inhibited by injection of ascorbic acid and thence deduced that ascorbic acid acts reversibly in the chemical reactions, especially the oxidation and the reduction processes, in a living organism, playing the role of the so-called biscatalysts, thus acting as activator or depressant to melanogenesis according to the chemical situation in the body. Accordingly, the results of our experiments may be consistently explained if we suppose that administration of ascorbic acid acts reducingly in the case of hypopigmentation, causing increase in serum tyrosine, while it acts in further expediting the oxidation of tyrosine in hyperpigmentation, causing diminution of the serum tyrosine value.

Fluctuation of Serum Tyrosine Following Adrenalin or ACTH Administration

Serum tyrosine measurements before, and 2–4 hours after, subcutaneous injection of 0.3 cc of 0.1% adrenalin were performed in 24 cases of pigmentary diseases, 27 cases of other skin diseases and 10 normal subjects. The vitiligo cases showed a rise in the serum tyrosine content, but the majority of the hyperpigmentation cases showed a decrease. But the normal controls and the patients with other skin diseases showed a general tendency to decreased tyrosine content.

Next, 25 mg of ACTH was injected on 8 cases of pigmentary diseases and 6 normal subjects and cases of other skin diseases, and their serum tyrosine content was measured 2 and 4 hours after injection. We see that the majority of the cases showed a reduction of the content, there being little difference observed between the normal controls and the cases suffering from abnormal pigmentation in this respect.

Discussion

Hisada *et al.*[21] and Kawahara *et al.*[22] have reported on the decrease of ascorbic acid in the adrenal glands following administration of adrenalin or ACTH, and Nishida[23] on the decrease of the same in the blood upon adrenalin injection. Carrié[24], in his animal experiments, found that the inflammation of skin aggravated by tyrosine administration was alleviated by administration of adrenal cortex hormone. The decrease of tyrosine in serum upon our application of ascorbic acid or ACTH in pigmentary diseases and other skin diseases may be interpreted as due to an improvement in the tyrosine metabolism by way of the adrenal function, and can be considered in collation with our clinical success in alleviating the pigmentation in some cases of Addison's disease and Riehl's melanosis by administration of adrenalin or adrenal cortex hormone.

Relation between Tyrosine and the Liver or the Thyroid Gland

In measuring the serum tyrosine contents in the above experiments, we did not neglect that the content may stand in correlation with the function of the liver, and in particular, with protein metablism, so that the urobilinogen content in urine and Takata's serum reaction were tested every time, but in the range of our experiments, no correlation worthy of special mentioning could be observed, excepting that among the patients having urobilinogen-positive urine or showing positive reactions to Takata's serum test, there were comparative many who showed high values of serum tyrosine in the summer season.

On the seasonal variation in basal metabolism there have been many reports, and the tendency of high values in winter and low values in summer has been generally accepted. This may be collated with the seasonal fluctuation of serum tyrosine we have reported above, and especially, the increase of serum tyrosine in the cases showing increased basal metabolism both in summer and winter, suggests a correlation between basal metabolism and serum tyrosine through the agency of the thyroid gland.

Ascorbic Acid in Blood of Pigmentary Diseases

The relation between ascorbic acid and the adrenal glands has long been drowing attention and the action of inhibiting dopa reaction by reducing quinone has been ascribed to ascorbic acid, but the relation between melanogenesis and ascorbic acid seems to stand on a more complicated mechanism. We tested the plasma of pigmentary diseases cases for the ascorbic acid content by the 2.4-dinitrophenyl-hydrazine (Leitz's photometer) method and obtained the results shown in Fig. 17. The values were subnormal chiefly in cases of acquired hyperpigmentation, such as Addison's disease, chloasma or post-inflammatory pigmentation. The cases of ephelides showed a rather wide range of the highest and the lowest threshold values. These results, together with the fluctuation of tyrosine values following ascorbic acid administration stated above, are interesting, and we hope for an opportunity to report on our proposed follow-up studies on this subject.

Summary

The author has examined the serum tyrosine content in 920 cases of dermatoses and normal controls. The results, especially seasonal variation of tyrosine content and its prominent change following sun-bathing have led us to infer that the precursor of melanin is supplied by blood tyrosine. The change of tyrosine content following ascorbic acid, ad-

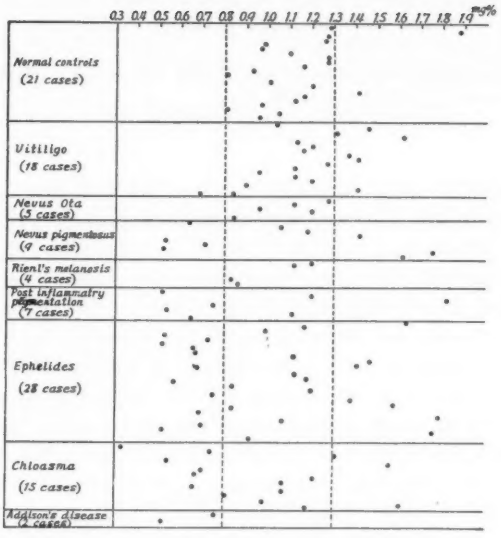
0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 mg%
Normal controls (21 cases)
Vitiligo (18 cases)
Nevus Ota (3 cases)
Nevus pigmentosus (9 cases)
Rienl's melanosis (4 cases)
Post inflammatry pigmentation (7 cases)
Ephelides (28 cases)
Chloasma (15 cases)
Addison's disease (4 cases)

Fig. 17. Ascorbic acid in the blood of pigmentary diseases

renalin or ACTH administration, suggested that a significance of adrenal functions in melanogenesis must be investigated from various points of view.

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VII. Serum Copper, Serum Oxidase and Melanin Formation

Since Keil and Nelson[1] noticed achromotrichia in black rats made deficient in copper experimentally, it has repeatedly been confirmed that the trace amount of copper is essential for the maintenance of fur color in many animal species. Although no comparable findings are reported in human, it seems fairly certain that copper is essential for the melanin production in man. Copper is indispensable for the activity of the enzyme tyrosinase, and according to Rothman *et al.*[2], copper reverses the inhibitory action of epidermal SH compounds on melanin production. So it is natural that many investigations have been made on the copper metabolism in clinical disorders characterized by abnormal melanin metabolism. The results obtained, however, are not always uniform. In the following, the concentration of copper in serum, the serum oxidase activity and their relationship in dermatological disorders are reported.

A. Serum Copper

Experimens and Results

The analytical method was similar to Gubler *et al.*'s[3] in principle. As the extinction of the copper-carbamate complex was rather low, the extraction of the final colored product with isoamylalcohol was found to be advantageous. Usual attentions were paid to avoid the contamination of metals.

The results are shown in Tables XI and XII.

Normal persons. 17 determinations were made (5 males and 12 females). No significant differences were niticed between both sexes. The mean was 102 μg/dl.

Pregnancy. In five pregnant women the average serum copper was 176 μg/dl and was significantly higher than normal.

Pigmentary disturbances.

Riehl's melanosis: In 9 cases of Riehl's melanosis the average was 111 μg/dl. In only two cases slight hypercupremia was observed.

Addison's disease: 4 cases. The mean was 128 μg/dl and the range was from 87 to 180 μg/dl. The slight increase in the average was not statistically significant.

Chloasma: 10 cases. None of them were pregnant at the times of the determinations. The mean was 101 μg/dl. In three cases the serum

TABLE XI
Serum Copper

Diseases	No. of cases	Serum copper (μg/dl) Mean±s.d.	Range
Normal controls	17	102±11.4	73–121
Pregnancy	5	176±59.9	115–255
Chloasma	10	101±24.9	72–145
Riehl's melanosis	9	111±19.4	80–137
Addison's disease	4	128±39.9	87–180
Postinflammatory pigmentaion	3	110±29.3	79–137
Ephelides	5	97±22.3	61–121
Lentigines	13	101±13.0	82–123
Nevus Ota	5	92±12.4	75–102
Nevus pigmentosus	7	96±11.6	82–113
Vitiligo	28	105±25.4	56–165
Leucoderma centrifugum	3	106±22.1	82–125
Juvenile canities	3	107± 6.6	82–125
Albinism	1	110	

TABLE XII
The Variation of Serum Copper
According to Age

Age (Years)	No. of cases	Serum copper (μg/dl) Mean±s.d.	Range
Under 5	5	135±26.4	111–172
5–9	5	143±29.1	95–170
10–14	8	102±20.8	84–147

copper was slightly elevated.

In postinflammatory pigmentation, ephelides, and in pigmented nevi there was no significant variation of serum copper.

Vitiligo: Serum copper was determined in 28 cases. The average was 105 μg/dl and the range was from 56 to 165 μg/dl. In three cases the values was below normal and in 10 above normal.

Leucoderma centrifugum: In 4 cases of Sutton's leucoderma, the mean was 106 μg/dl quite corresponding with that in vitiligo.

In juvenile canities and in albinism, the serum copper was within normal limit.

Other dermatoses.

In other dermatoses examined for comparison, significant increase in

serum copper level was noticed only in erythema nodosum. Although relatively high values were observed in erythema induratum, the elevation was not statistically confirmed and individual variations were large.

In other dermatoses including eczema, dermatitis, drug eruption, erythema exsudativum, chronic discoid erythematodes, pityriasis rosea, psoriasis, furuncle and herpes zoster, no significant variations in serum copper level were noticed.

Serum Copper in Younger Age Groups.

The cases other than pregnancy, allergic and inflammatory diseases were divided into several age groups and their mean copper levels were calculated (Table XII). In children below 10 years the serum copper tended to be high than the group above ten years of age. In the age group from 5 to 9 years the average was significantly higher than the normal adult value. Consequently, the cases below 10 years of age are not included in Table XI.

Summary of the Results.

Significant elevation of serum copper was observed only in pregnancy, and in erythema nodosum. In pigmentary disturbances, the values obtained were mostly within normal range except for several cases of Addison's disease. In childhood, especially between 5 and 9 years of age, the serum copper level was higher than in the adult.

B. Serum Oxidase

As shown in the previous section, the concentration of copper in serum did not change significantly in pigmentary dermatoses. Serum copper is not in the ionic state but is attached to α_2-globulin fraction of serum protein. It is known that this copper-protein has the activity of an oxidizing enzyme. So the serum oxidase activity is the functional manifestation of serum copper. In this section the activity of serum oxidase was determined.

Experiments and Results

The activity of serum oxidase was determined manometrically using Warburg apparatus with paraphenylenediamine as substrate. The method was essentially same with that of Hornykiewicz and Niebauer[4] with the modification that the buffer used was 1/5 M acetate buffer pH 5.9 in place of original phosphate buffer. The activity was expressed as maximum O_2 uptake (μl)/hr/0.5 ml of neutralized serum.

The results are summarized in Tables XIII and XIV.

Normal persons. 18 cases (8 males and 10 females). In 8 of them 12 monthly determinations were made for each individual. The variation from individual to individual was rather large. However, the month to

TABLE XIII
Serum Oxidase

Diseases	No. of cases	Serum oxidase (μl O_2/hr.)	
		Mean±s.d.	Range
Normal controls	18	44.6± 9.01	26.4– 66.1
Pregnancy	3	87.5±24.87	61.5–108.4
Chloasma	16	47.3±11.18	31.4– 61.9
Riehl's melanosis	9	56.2±12.18	40.9– 77.8
Addison's disease	5	58.8±26.58	36.6–103.4
Postinflammatory pigmentation	5	42.3±11.30	29.4– 60.0
Nevus Ota	10	44.2± 8.79	27.2– 56.2
Nevus pigmentosus	1	39.7	
Vitiligo	18	41.8±10.79	25.6– 65.9
Leucoderma centrifugum	3	39.3± 2.19	37.8– 41.8
Juvenile canities	2	26.6, 43.7	
Albinism	1	48.4	

TABLE XIV
Serum Oxidase in Younger Age Group

Age (Years)	No. of cases	Serum oxidase (μl O_2/hr.)	
		Mean±s.d.	Range
Under 5	8	65.1±12.87	49.4–85.5
5–9	7	62.5± 8.09	56.7–78.2
10–14	8	56.2±11.99	40.9–73.9

month variation was relatively small. The means in both sexes were almost identical, *i.e.* 45.8 in the male and 43.6 in the female.

Pregnancy. In three pregnant women the mean oxidase activity was 87.5 and significantly higher than normal.

Pigmentary disturbances.

Riehl's melanosis: In 9 cases of Riehl's melanosis the average was 56.2. This increase was statistically significant.

Addison's disease: 5 cases. The highest value was 103.4 and the lowest, 36.6. Although individual variations were large, the mean oxidase activity was significantly elevated.

Chloasma: 16 cases. No case was pregnant at the time of the determination. The mean was 47.3 and the values were all within normal range.

In postinflammatory pigmentation and in nevus Ota there was no change in the average oxidase activity.

Vitiligo: In vitiligo serum oxidase was measured in 18 cases. The average was 42.5 and was essentially same with normal value. No abnormally high nor low values were observed.

Leucoderma centrifugum: Three cases. The average was 39.3.

In juvenile canities and in albinism the serum oxidase activity was within normal limit.

Other dermatoses.

Among other dermatoses examined, significant increase in the serum oxidase activity was observed in erythema nodosum, cutaneous tuberculosis and in malignant melanoma. While in eczema, dermatitis, erythema exsudativum, chronic discoid erythematodes, and in pityriasis rosea, no significant increase was noticed. In seborrheic dermatitis and in scleroderma there was no consistent change in the activity.

Serum oxidase in childhood.

In children without pigmentary and inflammatory diseases the serum oxidase activity was higher than that of the adults (Table XIV). The highest value was noticed in the age group of under 5 years. But in 10 to 14 years of age, the activity was significantly higher than the normal adult value. Consequently, no cases under 15 years of age are included in Table III.

Summary of the results.

Significant increase in the activity of serum oxidase was observed in pregnancy, Riehl's melanosis, Addison's disease, erythema nodosum, cutaneous tuberculosis and in malignant melanoma. In vitiligo and in Sutton's leucoderma there was no significant decrease in the activity. In children under 15 years of age, the activity was higher than in adults.

C. The Relation between Serum Oxidase and Serum Copper

The results obtained in Sections A and B show that serum copper and serum oxidase do not reflect the pigmentary change in vitiligo.

In this section, the relationship between these two factors is studied.

Method and Results

In the following three groups, *i.e.* normal, vitiligo and melanosis groups, the correlation coefficients between serum oxidase and serum copper were calculated and the regression equations of the former to the latter were obtained assuming that the regression is linear. The melanosis group consists of chloasma, Riehl's melanosis and Addison's disease.

The result is shown in Table XV. The correlations coefficients ob-

TABLE XV

The Relation between Serum Oxidase and Serum Copper

Group	No. of cases	Serum oxidase (μlO_2/hr)	Serum copper (μg/dl)	r*	Regression† equation
Normal	9	41.7	89	+0.74	X=0.41Y+ 5.10
Vitiligo	23	42.5	100	+0.58	X=0.22Y+22.99
Melanosis‡	27	52.9	99	+0.85	X=0.46Y+ 7.42

* Correlation coefficient between serum oxidase and serum copper. † Regression equation of serum oxidase (X) to serum copper (Y). ‡ Total of Riehl's melanosis, Addison's disease and chloasma.

tained for these three groups were all significant at 5% level. It is observed that the coefficient is low in vitiligo. The regression coefficient of the equation regression is lowest in vitiligo group.

COMMENT

Since the experiment of Keil and Nelson, it has repeatedly been demonstrated that the trace amount of copper is essential for physiological melanin production. Cunningham[5] observed that the copper content of the colored skin was higher than that of the white skin of the animals of same species, and that the oxidation of dopa in the presence of aqueous extract of the skin was accelerated by the addition of cupric sulfate. Sarata[6] and Yoshikawa[7] reported that the colored skin of the mottled animal was richer in copper than the white part of the same animal and that human black hair contained larger amount of copper than the gray hair. According to Narasaka[8], copper was more concentrated in Mongolian spot than in the adjacent skin. Using gray Wien rabbit, Schuppli[9] observed increase in pigmentation after injections of cupric salt.

In the biochemical study of melanosis, Panlais *et al.*[10] noticed that copper of whole blood was high in Riehl's melanosis. In one of the cases examined by these authors the hypercupremia was of extreme degree amounting to 630 μg/dl.....nearly sixfold increase than normal. Volland[11] reported hypercupremia in melanodermas of nutritional origin and Liebetrau[12] noted increase of serum copper in chloasma and in Riehl's melanosis. But the results of above-mentioned investigators do not agree in the details. For instance, according to Paulais *et al.* the cupremia was normal in cases of vagabonds skin in contrast to Volland and in pigmentation faciale no hypercupremia was observed in contrast to Liebetrau.

In this investigation no significant increase in the concentration of serum copper was observed in dermatoses with increase in the melanin content of the skin including chloasma and Riehl's malanosis, even in the

evolutional stages. In Addison's disease, the slight increase was not statistically confirmed. It may be concluded that in our race at least, serum copper does not reflect the increase or decrease of the melanin.

This circumstance tells us that the alteration of copper metabolism in pigmentary disorders, be there any, will not be detected by the determination of copper in the serum. In this respect the present authors tried to know the functional state of serum copper.

It has long been known that the serum contains a factor with oxidative enzymatic activity. Hizume[13] noticed the increase of serum oxidase activity in pregnancy and surmised that this might be related to the hyperpigmentation in pregnancy. Melczer[14] reported higher values of serum oxidase in dark-skinned gypsy than in white race. Takigawa[15] and Nagaishi[16] reported that in vitiligo the activity was lower than normal. More recently Shacter[17] noticed the presence of a factor in serum which catalysed the aerobic oxidation of catechol and called this serum catecholase. His data suggested that the active group of this factor was copper. Hornykiewicz and Niebauer came to the same conclusion. Holmberg and Laurell[18]-[20] isolated a copper protein complex from blood serum and called this ceruloplasmin. This substance, in contrast with hemocuprein of Keilin and Mann, had enzymatic activity and catalized the aerobic oxidation of several phenolic compounds. According to Holmberg and Laurell the serum copper existed mostly, if not all, in the form of ceruloplasmin. This was later confirmed by Wintrobe and collaborators. Thus the serum oxidase activity is the functional manifestation of serum copper.

In this respect it is interesting that in this report high values of serum activity was observed in dermatoses characterized by the increase of melanin content of the skin, especially in Riehl's melanosis and Addison's disease although there was no corresponding significant increase in the serum copper level.

While on the other hand, there was no decrease in the serum oxidase in vitiligo. However, the correlation coefficient between serum oxidase and serum copper was lower in vitiligo than normal. This suggests the functional impairment of serum copper in vitiligo. In line with this the regression coefficient of the regression equation was lowest in vitiligo. This may be interpreted in two ways, either as the increase of non-ceruloplasmin copper as is observed in Wilson's disease or as the increase of inhibitors in serum. This will be discussed in the other article (p. 58).

What is the role of the increase of serum oxidase in the pathogenesis of melanosis? It is difficult to assume that ceruloplasmin directly participate in the melanin production. There are certain differences between ceruloplasmin and tyrosinase in the molecular weight and in the copper

content per molecule. Ceruloplasmin does not catalize the oxidation of tyrosine even in the presence of dopa or paraphenylenediamine. In albinism there was no change in the serum oxidase activity. So it appears that the serum oxidase has nothing to do with the physiological melanin production, as suggested by Lerner. But it must be stressed that ceruloplasmin as well as tyrosinase belong to the group of copper enzymes. Their activities are affected by substances that combine with copper. In this respect, the activity of ceruloplasmin is the better measure for the activity of tyrosinase than serum copper. Further, there remains the possibility that ceruloplasmin as a copper compound acts as the precursor of the tyrosinase of the skin.

Finally the authors pay attention to the fact that in our race the serum copper and serum oxidase activity were higher in childhood than in the adult. The same observation was made by Sarata[21]. In foreign literatures the serum copper level in infancy and childhood is reported to be identical with that of the adult except for a short period after delivery[22]-[24]. Referring to this respect, it is interesting to note that Leschi[25] reported higher copper level in adult Negro than in white race. This may be related to the abundance of melanin in colored race.

Summary

1. Serum copper level was significantly elevated in pregnancy and in erythema nodosum. In no diseases characterized by the abnormality of the melanin content of the skin, there was significant change of the copper content of the blood serum although in Addison's disease a relatively high value was noticed.

2. Serum oxidase activity was significantly elevated in Riehl's melanosis, Addison's disease, pregnancy, erythema nodosum and in cutaneous tuberculosis. In vitiligo and albinism the activity was normal.

3. The relationship between serum copper and serum oxidase was discussed. In vitiligo, abnormality in the correlation between these two factors was observed.

4. In infants and children the serum copper and serum oxidase activity tended to be higher than in adult.

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VIII. Copper Contents of the Epidermis and Corium

In the study of serum copper and serum oxidase in pigmentary disturbances, Tanji *et al.*[1] came to the conclusion that the serum copper *per se* could not be a measure for the melanin metabolism. It was stressed that the serum oxidase activity in relation to serum copper gave clue to the understanding of the role of copper in melanogenesis.

However, the concentration of copper in serum may not always parallel that in the skin, the actual site of melanogenesis. It is desirable to study the copper metabolism of the skin itself.

In this paper the copper content of the skin was studied and the results were correlated with the melanin content.

Materials and Methods

The skin specimens were mostly the skin of the axillary region removed surgically for the treatment of osmidrosis.

The specimens were grossly freed from the adipose tissue. The epidermis and the corium were separated by heating to 50°C. The separated epidermis and corium were thoroughly linsed with glass-distilled water, dried to constant weight in the oven at 100°C and weighed. Then the specimens were digested with sulfric acid and perchloric acid. The copper content of the clear digests were determined with diethyldithiocarbamate method. The copper content was expressed as mg copper/100 g dry tissue with standard deviations.

Small pieces of the same specimens were fixed in formaldehyde, mounted in paraffin, cut at 5 microns and were stained with Bodian's reduced silver method. ,According to the melanin content, the specimens were divided in Groups I to IV in the decreasing order[2]. The example of each group is shown in Figs. 18~21.

Results

Axillary skin. All specimens belonged to Groups II–IV. In the total 33 determinations were made (Table XVI). The copper content of the epidermis was always higher than that of the dermis. The average copper content was 3.957 in the former and 1.247 in the latter. No distinct difference was noticed between males and females. The mean copper content of each group was: in Group II, 3.636 ± 1.29; in Group III,

Fig. 18. **Group I.** Bodian's silver method. (100×)

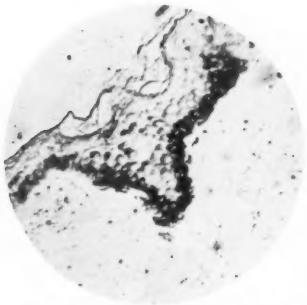

Fig. 19. **Group II.** Bodian's silver method. (100×)

Fig. 20. **Group III.** Bodian's silver method. (100×)

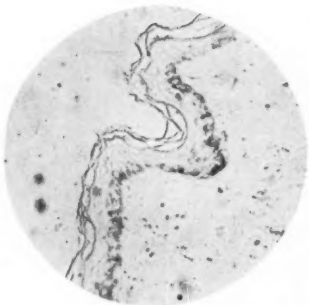

Fig. 21. **Group IV.** Bodian's silver method. (100×)

TABLE XVI

Regions	Group	Copper (mg/100 g dry tissue)			
		Epidermis		Corium	
		No. of determ.	Mean±s.d. (Range)	No. of determ	Mean±s.d. (Range)
Axilla	II	6	3.636±1.29 (5.705–1.943)	9	1.474±0.65 (2.617–0.701)
	III	13	3.577±1.15 (5.496–1.639)	15	1.162±0.38 (2.140–0.636)
	IV	7	4.475±2.780 (8.251–1.116)	9	0.961±0.32 (1.452–0.501)
Scalp	II	2	7.058, 5.657	1	0.897
Prepuce	I	3	3.028±1.56 (3.960–1.244)	3	1.236±0.64 (1.652–0.506)
Abdomen	II	1	5.592	1	1.202
	III	2	4.740, 3.616	2	1.339, 0.698
Thorax	I	2	4.670, 3.317	2	1.910, 1.423
	III	1	5.959	2	0.794, 0.493
Popliteal fold	II	2	4.645, 1.260	2	1.062, 0.636
Scrotum	I	1	7.574	1	1.170
	II	1	0	1	0.682

3.577±1.15; in Group IV, 4.475±2.78. That of the corium was: 1.474±0.65 in Group II; 1.162±0.38 in Group III and 0.961±0.32 in Group IV.

Skin of other regions. In three specimens of preputial skin, which all belonged to Group I in melanin content, the copper concentration was lower than that of the axillary skin. Thus the avarage was 3.028±1.56 for the epidermis and 1.236±0.64 for the corium.

In a case of scar tissue of the scalp in a five years old boy, the two determinations gave the results of 7.058 and 5.657 for the epidermal copper.

In a case of compound nevus of the leg, the copper content of the epidermis was 5.197 and 4.503 and that of the corium was 1.233 and 1.911 on two occasions. In another case of hairy nevus of the forehead, the copper content of the epidermis and the dermis were 3.881 and 1.652 respectively.

Effect of Ultraviolet irradiation.

In two cases of axillary skin, one side was excised after intense ultraviolet irradiation (30 cm, 10 minutes, 5 or 6 times). The copper content of the irradiated side was lower than the non-irradiated side. (Table XVII)

As it was difficult to obtain skin samples freely from patients, the following materials were obtained from cases autopsied at the Department of Pathology. As the cases succumbed to various diseases, the results may

TABLE XVII

Case No.	Irradiation	Copper (mg/100 g dry tissue)		Group
		Epidermis	Corium	
1	No irradiation	4.332	1.072	
	30 cm, 10 min., 5 times	3.108	0.644	
2	No irradiation	3.010	0.960	IV
	30 cm, 10 min., 6 times	1.521	0.840	IV

not represent the physiological values for respective regions.

Abdominal skin (three cases). The epidermal copper was 4.694± 0.99, dermal copper was 1.079±0.34. The first value was slightly higher than that of the axillary skin.

Thoracal region (three cases). The mean epidermal copper was 4.646±1.31 and the dermal copper was 1.155±0.64. The epidermal copper was again higher than in axillary region.

Popliteal skin. The results of two determinations were 4.645 and 1.260 for the epidermis and 1.063 and 0.636 for the corium.

Scrotal skin (2 specimens). The copper content of the epidermis was 7.574 in one case and 0 in the other case. That of the dermis was 1.170 and 0.682 respectively.

COMMENT

The copper content of the skin in relation to melanin content has been investigated by relatively limited numbers of investigators. Cunningham[3] observed that the pigmented skin of the animal was generally richer in copper than the white skin of the same species. According to Sarata[4], the colored part of the mottled animals contained larger amount of copper than the white skin of the same animal, and this was later confirmed by Flesch[5]. Narasaka[6] reported that copper was more concentrated in Mongolian spots that in adjacent skin. In pathological states, Paulais *et al.*[7] noticed about tenfold increase of copper in pigmented skin in a case of Riehl's melanosis. While on the other hand Robert[9] observed no decrease of copper in vitiligo patches. However, no study has been made on the relation between the physiological variation of the melanin content of the skin and the concentration of copper.

The results of present study showed that copper was almost invariably more concentrated in the epidermis than in the corium in confirmation of Flesch's results. This may be related to the fact that the physiological site of the melanin production is the epidermis.

However, the epidermal copper varied in a wide range even in the

same region. Moreover, no consistent high values were found in the skin areas physiologically rich in melanin. Thus the copper content of the preputial skin was lower than that of the axillary skin. In samples of axillary skin, the epidermal copper was highest in Group IV, although in dermal copper rough parallelism might be assumed with melanin content.

So it may be concluded that copper concentration does not vary in parallel with melanin content. Such results may be explained by the multiplicity of the factors concerned in the physiological melanogenesis. The aim of further study should be to investigate the functional state of copper in the skin. In this respect it is interesting to note that in two cases the copper content of the epidermis and dermis decreased after ultraviolet irradiation. Although the small numbers of cases do not allow to draw conclusion at present, further study in this line may give clue to the understanding of the role of copper in melanogenesis.

Summary

The copper content of the epidermis and the corium was determined.

The copper concentration was higher in the epidermis than in the corium.

There was no correlation between the copper content of the epidermis and the melanin content.

After ultraviolet irradiation, the skin copper tended to be low.

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IX. Copper-Iron Iontophoresis Treatment of Vitiligo

In the action of tyrosinase—a complex of copper (0.2–0.3%) and protein—copper has been deemed indispensable for its enzymal activity, by Kubowitz[1], Keilin and Mann[2]. Observations on the correlation of copper and the colors of the skin and the hair have been reported by Keilin and Nelson[3], Cunningham[4], Gorter and others[5], and also by Sarata[6] and Yoshikawa[7] of this University. Flesh[8] made a follow-up study of the results of Sarata and found that the copper content is larger in the epidermis than in the corium and that the oxidizing reaction of dopa is most effectively expedited by copper of all metals. In vitro experiments, Robert[9] also confirmed that copper and iron promote the dopa reaction. Schuppli[10] succeeded in expediting oxidation by adding sulfate of copper or iron to dopa solution, and in producing hyperpigmentation by a single injection of 1% aqueous solution of cupric sulfate intracutaneously in Wien rabbits. Liebetrau[11] and Schuppli[12] have pointed out an increase of blood copper in hyperpigmentary diseases and in pregnancy and Gracianski[13] an increase of blood copper and skin copper in Riehl's melanosis. Figge[14], Rothman[15] and Flesch gave the interpretation that the -SH groups in the skin inactivate tyrosinase by combining with the copper in it. Iijima and Tokunaga[16], in their experiments on guanofuracin leucodermia, confirmed the fact that the inhibitive action of guanofuracin against the enzyme is nullified by addition of cupric sulfate, whence they inferred the occurrence of chemical reaction between copper and tyrosinase, and concluded that the copper of the enzyme tyrosinase is not only the activity center but also the binding point of the enzyme to its substrate. On the other hand, it is well known that iron also acts as activating radical in reducing or oxidizing enzymes or as simple catalyst. It has drawn much attention, in connection with cytochrome and peroxidase and since the days of Warburg, its effect in activating intracellular oxidation has been pointed out. In particular Katsunuma[17] has demonstrated that indophenol-blue reaction is brought out by the catalysis, cellular iron. Robert, Zürcher and Schmidli[18] have reported an increase of iron and copper contents in hyperpigmented skin, Stüttgen[19] observed an increase of melanin in hemochromatosis, and in a case of the disease experienced by Matsumoto[20] of our clinic, melanin granules of the epidermis was found also slightly increased.

Our Copper-Iron Iontophoresis Treatment

For treating vitiligo Shaffer[21] tried intracutaneous injection of a

0.1% cupric sulfate solution and external application of cupric sulfate ointment, but gave up the attempt as fruitless. Our clinic[22], however, has succeeded in achieving an unpreceded success in treating leucoderma by iontophoresis based on an utterly new idea.

The method is as follows: 1) Effect electrolysis with iron needles as anodes planted in about 2 mm intervals on the leucoderma foci. Direct current below 2 mA strong is sent through the needles inserted to the depth of 1–1.3 mm in the skin and the treatment is repeated in succession over the whole focal area, for 5–10 seconds per spot. 2) Next, cover the focus with gauze soaked in a saturated cupric sulfate solution or apply 10% cupric sulfate hydrophilic ointment on it, place a carbon rod thereupon as anode and apply direct current of 0.5 mA per square cm for 5–10 minutes. The focus will appear light yellow-green and the points where the needle has been inserted somewhat bluish following the treatment. 3) Immediately irradiate the treated focus with artificial sunlight at a distance of 30 cm for 10 minutes. Repeat this treatment approximately once per week.

Progress: About one week after the first treatment, the needle pricks will be found covered with petechial crusts or bloody crusts and with red halos around them. In another week the crusts have already fallen off and small dots of a pin-head size of rusty yellow or inflammatory scarlet color and of orange-brown color probably indicating generation of melanin are found sporadically. Gradually the areas of brownish tone spread out centrifugally from these dots or the hair follicles outwards, fuse together and become diffusive, the rusty color fades out and the whole area of the focus recovers a nearly normal skin color, and pigment is observed to invade into the involved area from the surrounding normal skin (Table XVIII & Figs. 22–27).

TABLE XVIII

Results of Cu-Fe-Iontophoresis Treatment

Chiefly affected \ Result	Healed	Improved	Slightly improved	Unchanged	Total
Whole body	0	8	2	1	11
Clothed sites	2	16	2	1	21
Exposed sites	11	30	2	7	50
Total	13	54	6	9	82

The number of rounds of the treatment required is not fixed, varying according to the size or the oldness of the focus, but in usual cases, 3–10 rounds are indicated. In the case of rapid progress, the focal area becomes

almost undistinguishable 3 months after the last treatment. Since iontophoresis of iron and copper separately showed a tendency to slacken the reappearance of skin pigment, iron and copper were used in combination, in a majority of the treated cases. In the cases of patients with vitiligo resembling acrodyschromatosis[23], electric cupric sulfate bath was administered at 3 mA for 30 minutes several times at one week's intervals, and we found the foundaries of the foci beginning to blur after 3 baths and the normal finding entirely restitutted in the 3rd month after the 9th and last bath. The results of this treatment were shown in Table XVIII, and Figs. 22–27.

Histological and Histochemical Examinations

Skin sections were sampled from 4 cases 2, 3 and 4 weeks and 3 months after the first treatment and subjected to hematoxylin-eosin staining, van Gieson's staining, dopa reaction, iron-copepr demonstration by Boyce and Herdmann's method (1.5% ferro-cyankali, 0.5% HCl $\widehat{aa}$) and copper demonstration by Okamoto's method[24]. The findings were as if in endorsement of the foregoing clinical process.

Results: In the section resected two weeks after the treatment, the blood vessels were found dilated, around them was observed a slight infiltration of round cells and histiocytes, and a positive iron reaction was found around the sites inserted by the needles. In the 4th week section, the said inflammatory sign was nearly gone away, the indigo color of iron had been diffused all over the corium and a part of the histiocytes were found perceptibly dark-stained to an indigo tone. In some sections, the lower half of the epidermis was stained light-blue. That was a picture of iron ions, introduced through the needle, diffusing out in the corium and the deeper layer of the epidermis, and particularly of those engulfed by histiocytes. In the 3rd week sections, however, brown granules strongly resembling melanin were found in the basal cells and in the fusiform or stellate histiocytes in the papillar or subpapillar layer. These granules showed a positive dopa reaction, were discolored by H_2O_2 and were negative to Berlin-blue so that they might be identified with melanin granules. In the sections treated by Boyce-Herdmann's and Okamoto's methods, we found shining light-red granules intermingled with the said melanin granules in the basal cells and in the histiocytes in the papillary layer and were presumed to be copper ions. The picture led us to an inference that it is not a case of mere tattooing with copper and iron, but of a nearly physiological production and distribution of melanin due to the oxidation expedited by the catalyzing action of these metals. Thus, the iontophoretic method is not only proposed herewith as a new method for treating vitiligo, but also as a promising means suggesting the importance

of the inactivation or the denaturation of the enzymes in the foci as a factor in the pathogenesis of vitiligo. Taking into consideration also the report by Fujino[25], Matsumoto[26] and Komatsu[27] stating that the serum tyrosine content in vitiligo cases does not decrease and the observations of Liebetrau, and Iijima and Tanji[28] to the effect that the blood copper content in vitiligo cases is not always reduced, we are led to the conclusion that this disease is not caused by a deficiency in the mother substances of melanin or blood copper etc., but that it is definitely a disease due to the local disturbance in the distribution or function of enzymes.

SUMMARY

New treatment method of vitiligo was presented and discussed. The method, consisted of copper-iron-iontophoresis and ultraviolet irradiation, brought a excellent effect. Basing on this good result, the pathogenesis of vitiligo was considered and it was concluded that vitiligo is caused by the local disturbance in the distribution or function of enzymes.

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Fig. 22. Before treatment.

Fig. 23. After treatment.

Fig. 24. Before treatment.

Fig. 25. After treatment.

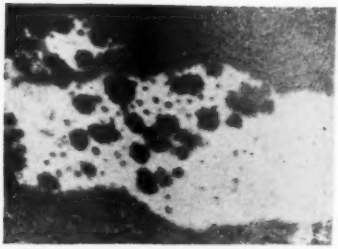

Fig. 26. Before treatment.

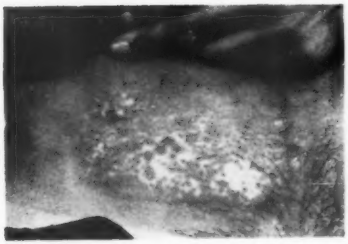

Fig. 27. After treatment.

X. Inorganic Metal Elements in the Skin Tissue and the Hair

Since the inorganic metal elements have the catalytic action in the photosynthesis of the plant chlorophyll, it is assumed that they play an important role in the oxidation-reduction reaction in living organisms. According to Thatcher's description, Cu and Zn or Fe and Mn, respectively, are the 'mutual conjugate catalysts' in the oxidation-reduction reaction. In view of this standpoint, we undertook the spectrographic analysis with emission spectrum of the skin tissue and the hair (by Quartz Spectrograph QF 60 Shimazu).

Experiments

The skin tissues of nevus pigmentosus (9 cases), vitiligo (2 cases) and normal persons (4 cases), and black hairs of adult women (6 cases), grey hairs of old men and woman with canities seniles, and white hairs of an albino (8 years old girl) were carbonized at 300°C–400°C. These specimens were burned in the hole of carbon electrode (depth 4 mm, diameter 1.5 mm). Conditions: Slit 0.03 mm, distance of electrodes 0.2 mm, 100 volt, 5–6 amp, time of exposure 10–12 seconds (Plate, Fuji AI, Development FD-31). Spectrums were determined by the wavelength tables of 100,000 spectrum lines (Harrison, G. R.).

Results

The skin of nevus pigmentosus showed more increased Zn and Mn than the skin of vitiligo. The black hairs showed more increaseed Zn and Mn, and more decreased Mo than the grey hairs.

These skins and hairs showed no significant difference in the other elements (Al, B, Ba, Cu, Fe, Mg, P, Si, Sn, Ti).

Comment

Sarata[1], Flesh[2], Graciansky[3] and Robert *et al.*[4] reported the increase of Cu in the pigmented skin tissues. Tanji[5] reported the copper concentration was higher in the epidermis than in the corium and after ultraviolet irradiation the skin copper tended to be low. Recently, Bowners and Morton[6] pointed out the presence of Zn in the pigmented tissues of eyes in fresh-water fihses. Kikkawa *et al.*[7),8] investigated relationships between skin or hair colors and metal elements from a genetical

point of view, and admitted that contents of Cu, Co, Fe, Mo, Ni and Ti in the hairs vary paperchromatographically according to the color of the hairs respectively.

In our experiments, Cu and Fe showed no significant difference between hyperpigmented and normal skins or black and grey hairs. But, Zn and Mn increased in hyperpigmented skin or in black hair.

Considering that Cu and Zn or Fe and Mn, respectively, are the mutual conjugate catalyst in the oxidation-reduction reaction, the results are very interesting for us. Moreover, the following experiments that the sheeps fed in Mo-containing pastures brought about depigmentation in the skin and hair, and this depigmentation was prevented by the supply of Cu, support our results that Mo is increased in the grey hairs.

Summary

Inorganic metal elements in the skin tissue and the hair were measured by Quartz spectrograph QF 60. Zn or Mn-mutual conjugate catalyst of Cu or Fe respectively-increased in hyperpigmented skin than in normal skin, and also increased in the black hair than in the grey hair. Mo increased in the grey hair than in the black hair. No other elements showed any significant difference.

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XI. Blood Sulfhydryl Contents and Melanin Formation

Recently there is an increasing interest in the function of sulfhydryl groups in animal body. SH group is essential for the activities of certain enzymes and for some vital functions of the cell. In dermatology, special attentions have been paid to the role of SH group in keratinization and melanogenesis. In this report the SH contents of the whole blood and blood serum in dermatological disorders, especially in pigmentary disturbances are reported.

Method

The method was the same with that reported by Bidmead and Watson[1]. The non-protein SH of the whole blood was determined amperometrically using rotating platinum electrode by titrating deproteinized hemolysate in supporting electrolyte with 0.001 N silver nitrate solution.

The same procedure was employed for the determination of protein-bound SH of the blood serum. 1 ml of serum was added directly to the supporting electrolyte solution. No protein denaturant other than ethyl alcohol was used.

All determinations were made in duplicate. The results of two determinations agreed within the error of ±5% in most cases.

Throughout the experiment, glass-distilled water was used for the washing of glasswares and for the preparation of the reagents.

Results

The results are summarized in Tables XIX–XXII.

Normal persons. In twelve healthy individuals (7 males and 5 females) between 19 and 34 years of age, the total of 61 and 71 determinations were made for the whole blood SH and protein bound SH respectively. The mean SH content of the whole blood was 101.0 μM/dl in accordance with the value reported by Bidmead and Watson. The serum SH was 66.6 μM/dl and was slightly higher than the values reported by other investigators. The SH of the whole blood was higher in the female and protein bound SH was higher in the male (Table XIX).

Seasonal variation. In five healthy individuals (four males and one female) the determinations were made at monthly interval throughout the year. The values obtained in the summer (July and August) were

lower than those obtained in the winter (December and January) (Table XX).

Pregnancy. In five pregnant women not complicating with other diseases the average SH content of the whole blood and blood serum was significantly lower than normal. This tendency became more apparent as the termin is neared.

TABLE XIX

Normal Persons

Sex	SH (μM/dl)					
	Whole blood			Blood serum		
	No. of determ.	Mean±s.d.	Range	No. of determ.	Mean±s.d.	Range
Male	49	98·8±12.1	80.0-126.8	55	67.7±6.7	43.0-83.0
Female	12	110.2±19.3	85.0-156.0	16	62.5±5.2	53.5-72.0
Male and female	61	101.0±14.4		71	66.6±6.7	

TABLE XX

Seasonal Variation of the SH Content of the Blood in Normal Persons

Season	No. of determ.	SH (μM/dl)			
		Whole blood		Blood serum	
		Mean±s.d.	Range	Mean±s.d.	Range
Summer*	10	96.2± 8.6	80.0-109.5	64.1±4.0	55.0-69.0
Winter†	10	107.5±11.8	90.0-128.0	66.0±4.0	61.5-73.0

* July and August. † December and January.

Pigmentary disturbances (Table XXI).

Riehl's melanosis: The determinations were made in 9 cases. The mean SH content of the whole blood and blood serum was essentially normal. However, it is interesting to note that in one case the values were low in the initial stage of the disease and became higher after a year when leucomelanodermia developed after the application of monobenzylether of hydroquinone.

Addison's disease: In one case the SH content of the whole blood and blood serum was slightly low and that of the whole blood was low in another.

Chloasma: In chloasma excluding chloasma virginum periorale, the

SH content of the whole blood and blood serum was lower only to slight extent. In chloasma virginum the value was slightly above normal.

In postinflammatory pigmentation, pigmented nevi and in freckles the whole blood SH was higher than normal while serum SH showed almost normal value. In two cases of malignant melanoma there was significant decrease in serum SH.

Vitiligo: The SH content of the whole blood was slightly higher than normal. While no correlation between the degree of the increase and the extent of the disease was observed, the whole blood SH was higher in cases with shorter duration of the disease (Table XXII).

TABLE XXI

Diseases	SH (μM/dl)					
	Whole blood			Blood serum		
	No. of determ.	Mean±s.d.	Range	No. of determ.	Mean±s.d.	Range
Normal controls	61	101.0±14.4	80.0-156.0	55	66.6± 6.7	43.0- 83.0
Pregnancy	5	72.5± 8.6	64.0- 87.0	5	54.7± 7.0	48.0- 66.0
Chloasma	9	98.3±14.0	72.0-112.0	10	63.8± 8.8	52.0- 80.5
Chloasma virginum	3	106.8±17.3	90.5-125.0	3	68.3± 3.7	66.0- 73.0
Riehl's melanosis	10	103.2±29.7	41.0-144.0	10	64.0± 7.9	50.0- 75.0
Addison's disease	2	95.5, 96.5		2	59.0, 66.8	
Postinflammatory pigmentation	6	116.3±17.3	90.0-139.0	6	67.6± 7.9	62.5- 73.2
Ephelides	3	106.3±13.3	90.0-118.0	3	66.2± 3.9	63.0- 70.5
Nevus Ota	6	101.2±19.2	85.0-138.0	5	69.0± 3.5	64.0- 73.0
Nevus pigmentosus	4	125.8±17.7	106.0-149.0	5	68.0± 4.8	60.0- 72.0
Vitiligo	16	109.7±13.3	88.0-133.0	18	68.1±10.0	54.0-101.0
Malignant melanoma				2	35.5, 47.5	

TABLE XXII

Whole Blood SH and Duration of the Disease in Vitiligo

Duration of disease	No. of cases	Whole blood SH (μM/dl)	
		Mean±s.d.	Range
1 month or shorter	5	118.2±19.6	92.0-133.0
2 months to 1 year	5	108.4±11.1	95.0-120.0
2 years to 10 years	5	107.6± 2.8	104.0-111.0
Above 10 years	1	88.0	

Comment

The melanin is generally believed to be the oxidation product of tyrosine by tyrosinase. Tyrosinase is a copper-protein complex and copper is essential for the activity of tyrosinase. Substances which combine with copper inhibit the activity. Among such substances organic sulfur compounds are most well known. Rothman *et al.*[2] proposed the theory that the SH compound in the epidermis is the natural inhibitor of melanogenesis. They found that the aqueous extract of the epidermis inhibited melanin production in vitro and that this inhibition is reversed by SH inhibitors such as copper and parachloromercuribenzoate. Further it was noticed that the inhibitory activity was the function of the SH content[3]. In clinical disorders it was demonstrated that the SH content was high in vitiligo patches and low in hyperpigmented skin[4]. Iijima *et al.*[5] observed that in vitiligo the activity of serum oxidase was low compared with serum copper and surmised that inhibitory substances were increased in the blood.

In dermatology, the investigations of previous authors on the SH content of the blood were mostly confined to the determinantions of glutathione of the whole blood and the results were mostly negative. In the present study, the SH content of the whole blood and blood serum was determined with an improved method.

As the metablism of SH compounds has not been wholly elucidated, and, moreover, as the function of SH compounds are multitudinous, it may be difficult to interprete the results obtained in dermatological disorders, which are often caused by unknown mechanism. The alteration of SH content of the blood, which may be detected, may be either the cause or the results of the diseases. With this limitation in mind, some of the results are discussed in the following, especially in reference to the problem of melanin production.

In contrast to the results obtained by Shiroishi[6], whole blood SH and protein bound SH were lower in summer than in winter, although only slightly in the latter. This finding, in common with the decrease of serum tyrosine level in summer, suggests some relationship with melanin production. The mechanism by which this reduction in SH content takes place is not easily explained. It is possible that increase in the environmental temperature or ultraviolet ray is responsible for the reduction. The effects of these two factors on SH compounds are variously reported. Extreme cold environment reduces the SH content of the liver in experimental animal[7]. While on the other hand the SH content of sera is reduced after heating[8]. According to Woodward[9], glutathione is oxidized by ultraviolet ray, while Goldblum reported the increase of SH level of blood serum after ultraviolet irradiation in vitro. Using histochemical

method, Percival and Stewart[10] observed no change in SH pattern of the skin. While most authors noted increase in the extractable SH of the skin after ultraviolet irradiation, the reverse was reported by Flesch. Although such discrepancies may be due to the difference in the methods of determinations or the species of animals used and to the difference of the dosage of irradiation, it is at present difficult to quote the experimental results for the explanation of present results. At the present stage of knowledges it is safe to assume that the decrease in SH in summer is secondary to the generalized increase in melanin production.

The decrease in the SH contents of both whole blood and blood serum was remarkable in pregnancy. Although it may be related to the anemia and hydremia of pregnancy, its relation to the increase in pigmentation cannot be overlooked. According to Figge[11], estrone relieses the inhibition of tyrosinase by SH compounds. The decrease in the SH together with the increase in the estrogen in the latter half of pregnancy plays an important role in the hyperpigmentation of pregnancy.

The same may be expected in chloasma other than chloasma virginum which had developed in most cases during pregnancy. The whole blood SH was low compared with the normal female value but not so markably as in pregnancy. Perhaps the laps of time after the onset has vagued the changes.

In Riehl's melanosis the values obtained were mostly within normal limit. However, it is interesting to note that in one case the initial whole blood SH was abnormally low and became higher after a year when leucoderma occurred after the use of monobenzylether of hydroquinone.

In Addison's disease the SH content was not so low as reported by Binet and Weller[12]. It may be due to the fact that the cases were under treatment with adrenal steroid and ascorbic acid.

The SH content of the whole blood was high in vitiligo. This is in accord with the increase of SH in vitiligo patches and suggests the important role of SH in vitiligo. Although higher values are obtained in other diseses such as postinflammatory pigmentation and pigmented nevi, the etiological relationship between SH and vitiligo is showen by the following facts. The increase of SH does not parallel with the extent of the disease. While the increase was most markable in cases with short clinical course and least so in long-standing cases. This suggests that the increase is not secondary to the cessation of melanin production but is in some ways related to the etiology of the disease.

In malignant melanoma the decrease of serum SH was remarkable. At present it is difficult to decide wheather this decrease is directly related to the excessive melanin production or not. The decrease of erythrocyte GSH was observed in a terminal case of melanoma by Tzanck *et al.*[13].

The high values observed in pigmented nevi and postinflammatory pigmentation are worthy to note. In the former disease, it is the impression of the author that the apparent high value is not directly related to the disease, becaues the determinations in this disease were done exclusively in winter when the SH value is physiologically high.

Summary

From these results, it may be said that in several pigmentary disturbances the variation of the SH content of the blood follows the same pattern as that of the melanin content of the skin. In some of them, *e.g.* in pregnancy and vitiligo the variation may have direct relationship with the dyschromia. While in others, no variation of SH in the blood was observed. It is of interest that the latter group consists chiefly of local malformation or of diseases restricted definite areas of the skin.

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XII. Monobenzylether-Hydroquinone Leucomelanodermia

Oettel[1] reported in 1936 that when hydroquinone was fed to black haired cats in doses of 30 mg. per kilogram daily over a period of six to eight weeks, that hair turned to grey. Nakajo[2),3] in our department reported in 1942 that hydroquinone injected on black goldfishes brought about fading of melanin in their epidermis and scales, and that the mechanism of depigmentation by hydroquinone could not be explained by mere reduction of melanin, but rather by the special suppression against dopa oxidase through its alkalization potency. Oliver *et al.*[4] first reported in 1939 that an antitoxidant known by the trade name of Agerite Albe in rubber gloves was responsible for depigmentation of the skin of workers wearing the gloves, and in 1940[5] that monobenzylether of hydroquinone in Agerite Alba had a characteristic effect on pigmentation. These reports suggested a possibility of clinical use of this compound for treatment of melanin hyperpigmentation. Since then, occasional reports have appeared on the efforts to use crude monobenzylether of hydroquinone therapeutically without success. Recently, Lerner and Fitzpatrick[6] reported that a good clinical response was shown in 64 of the 84 patients with increased melanin pigmentation treated with 20% monobenzylether of hydroquinone (a purified and finelly dispersed concentrated preparation). These good results have led many dermatologists in Japan including the present author to use the agent for treatment of hyperpigmentation. And many of them[7),8),9),10] reported that a good result was obtained by the agent in our race too. The present author[11], however, discussed this problem from a different point of view that pigmentary response in our race may be differ from that in the White or the Negro races and advised that the agent should be used for treatment only by prescription of the dermatologist. Unfortunately in Japan, cosmetics containing monobenzylether of hydroquinone are perimitted to be put on general sale and have been good sellers. In 1956 a characteristic leucomelanodermia was diagnosed in the face of a patient who visited our clinic and was ascribed to the administration of monobenzylether of hydroquinone. Since that time, this type of disease has been occasionally met with not only in our clinic but by many other dermatologists in Japan. This paper is a report of 12 such cases examined in our clinic during 1956.

Report of Cases

Case 1. Y. S., a housewife, aged 33, had chloasma in the cheeks of ap-

proximately four years' duration, had been treated with monobenzylether of hydroquinone by a physician since July 1955. One month's therapy showed some lightening of the skin color. But after three months' therapy, numerous depigmented spots were present on the cheeks in small sizes, up to 2 or 3 cm in diameter, and in irregular or round shapes, and nondepigmented skin on these regions varied from a deep brown to a dirty slate color. The continued treatment made the depigmented area increased in size and the pigmented area more hyperpigmented. In January, 1956, she was first seen in our clinic. Leucoderma was found on the nose, lower eyelids, inner sides of the cheeks, around the mouth and the chin, and diffuse, deep brown to slate colored, hyperpigmentation mingled with reticulated or small-island-shaped depigmented spots was found in the outer sides of the cheeks. Thus, this case showed two kinds of pigmentary change: 1) vitiligo-like change. 2) hyperpigmentation mingled with depigmented spots in a reticular arrangement. But, it was clear that the former lesion was predominant in this case. This type of leucomelanodermia was named " depigmented type " by the present author. Laboratory findings: Blood pressure 128–82 mm Hg; Aschner's phenomenon, negative; basal metabolic rate +17%; Thorn's test (epinephrine) −85%; serum tyrosine 2.14 mg/dl; serum sodium 142.2 mE/l; serum potassium 4.75 mE/l; potassium tolerance test +40% (abnormal); urine, normal.

Case 2. S. T., a housewife, aged 30, was dark-complexioned. Since November 1955, she had applied a cosmetic containing 1.5% monobenzylether of hydroquinone for the purpose of becoming a fair-complexioned. In January 1956, she noticed a depigmentation on the central region of the face and continued the application of the cosmetic more frequently. Then depigmented areas increased in size symmetrically and expanded over the forehead, the nose, inner sides of the cheeks, around the mouth. At the same time, diffuse brown-slaty pigmentation appeared on the outer sides of the cheeks. She discontinued the application of the cosmetic in April, but the depigmented area increased in size and the hyperpigmented area became gradually darker in color. She came to our clinic in May 1956. She gave similar findings as in Case 1 (Fig. 28). No inflammatory signs could be found. This case seemed to belong to the " depigmented type " of leucomelanodermia. Laboratory findings: Blood pressure 120–70 mm Hg; Aschner's phenomenon, positive; basal metabolic rate +18%; Thorn's test (epinephrine) −67%; serum sodium 142 mE/l; serum potassium 4.6 mE/l; potassium tolerance test +47.8% (abnormal); urine, normal. Course: Injections of ascorbic acid and adrenal preparation combined with internal use of these medicines resulted a moderate improvement. After treatment of about six months, the depigmented area decreased in size and mingled with normal pigmented island-shaped spots, each of which had its origin in the hair follicules. Thus, about half the depigmented area could be recovered.

Case 3. C. A., a housewife, aged 41, had applied a cosmetic containing monobenzylether of hydroquinone with the purpose of treating chloasma-like pigmentation on the face since June 1955. In November, two round, fingertip-sized depigmented spots were present on the forehead, and in December erythema

and swelling suddenly appeared on the face twice, and each time disappeared after a week's symptomatic treatment. In April 1956, the cosmetic applied continuously brought on numerous, variously shaped and sized, depigmented spots on the face. Borders of the white spots and nondepigmented area, which localized on the forehead and the nose, were hyperpigmented in dark brown color. Nevertheless, she consulted no doctor and applied the same cosmetic more earnestly. In October 1956, when she first visited our clinic, she showed severe symptoms of " depigmented type " of leucomelanodermia (Figs. 30, 31). Laboratory findings: Blood pressure 110–60 mm Hg; Aschner's phenomenon, positive; basal metabolic rate +14%; Thorn's test (epinephrine) −84.6%; serum sodium 139 mE/l; serum potassium 4.6 mE/l; potassium tolerance test +41.3% (abnormal).

Case 4. S. T., a housewife, aged 27, had had chloasma (pregnancy) in the regions around the eyes and around the mouth since May 1954. She had since been applying a cosmetic containing monobenzylether of hydroquinone till September 1956. In July 1956, numerous depigmented spots and diffuse hyperpigmentation were present on the face and neck. These symptoms were gradually aggravated. In November 1956, she was first seen in our clinic, showing a " depigmented type " of leucomelanodermia on the face and neck. Laboratory findings: Blood pressure 130–70 mm Hg; Aschner's phenomenon, negative; basal metabolic rate +21%; Thorn's test (epinephrine) −25%; serum sodium 144.0 mE/l; serum potassium 4.4 mE/l; potassium tolerance test +56.8% (abnormal).

Case 5. M. M., a housewife, aged 35, had freckles of exposed areas since childhood. She applied a cosmetic containing monobenzylether of hydroquinone (a different cosmetic from the above-mentioned) for about a year. During the first two months, she had several fits of erythema and swelling on the face. But, none of such fits occurred after three months' application. After eight months, brown-slaty pigmentation was present on her face especially on the cheeks. Closer observation revealed the presence of samll spots of depigmentation in the cheeks. Nevertheless, she did not give up using the cosmetic. Four months after the onset of pigmentary disturbances, she was first seen in our clinic. On the forehead, a depigmented zone of 2–3 cm in width could be found. The most prominent finding, however, was obtained on the cheeks. There was found diffuse dark-brown-slate colored hyperpigmentation mingled with relatively depigmented small spots from one to several mm in diameter. On closer observation, some of the depigmented spots presented a reticular arrangement. Thus, an abnormal contrast between hyper- and depigmentation brought about such an extreme disfigurement of the face that she was almost suffering from secondary neurosis. This type of leucomelanodermia was named " reticulated type " by the present author. Laboratory findings: Blood pressure 133–76 mm Hg; Aschner's phenomenon, positive; basal metabolic rate +14%; Thorn's test (epinephrine) −67%; serum sodium 144 mE/l; serum potassium 4.1 mE/l; potassium tolerance test +92.8% (abnormally high value); urine, normal. Course: Injections and internal uses of ascorbic acid and of adrenal preparation brought about a great improvement. After the treatment

for two months, small depigmented spots on the cheeks became repigmented and depigmented area on the forehead decreased in size and degree, but the brown-colored skin remained unchanged. After the four months' treatment, the pigmentary disturbance was perfectly healed. At the time, laboratory tests revealed that serum sodium was 153.0 mE/l and sodium potassium 3.0 mE/l, but no potassium tolerance test was carried out because of vomitting.

Case 6. T. S., a housewife, aged 31, had had post-traumatic pigmentation on the right cheek since June 1955. She was treated by monobenzylether of hydroquinone by a physician. After two months' therapy some lightening of the skin color was seen. In January 1956, dark brownish pigmentation appeared diffusely not only on the right cheek but also on the left mingled with many small depigmented spots. The treatment was continued with no improvement but rather with aggravation. In August 1956, she was first seen in our clinic. A typical " reticulated type " of leucomelanodermia was noticed as in Case 5. Laboratory findings: Blood pressure 120–80 mm Hg; Aschner's phenomenon, positive; basal metabolic rate +13%; Thorn's test (epinephrine) −50%; serum sodium 148 mE/l; potassium 4.2 mE/l; potassium tolerance test + 27.2% (normal). Course: The similar treatment as in the above-mentioned cases for about two months brought about moderate improvement. The depigmented spots recovered that normal skin color but diffuse brownish hyperpigmentation still remained. After three months' treatment, it became very difficult to differentiate this case from a mild case of chloasma. At the time laboratory tests revealed that serum sodium was 137 mE/l, serum potassium 5.7 mE/l and potassium tolerance test +24.4% (normal).

Case 7. M. O., an office girl, aged 22, had freckles on the face since childhood. She was treated with 5–10% monobenzylether of hydroquinone for eight months with much improvement. Continued application, however, brought about darkbrowish or brownish-slaty pigmentation mingled with many rice-grain-sized depigmented spots on the cheeks, some of which were connected with each other in a reticular arrangement. This case was diagnosed as a " reticulated type ". Laboratory findings: Blood pressure 132–70 mm Hg; Aschner's phenomenon, negative; basal metabolic rate +28%; Thorn's test (epinephrine) −72%; serum sodium 144 mE/l; serum potassium 4.1 mE/l; potassium tolerance test +47.8% (abnormal); erythrocyte count 3,500,000, leucocyte count 5600 (polymorphonuclear 44%, lymphocytes 46%, monocytes 2%, eosinophils 8%); blood sedimentation rate 5 mm (1 hour); histamine scratch test, 130 seconds in a depigmented site and 90 seconds in a hyperpigmented site of the sideneck, and 160 seconds in a depigmented site and 100 seconds in a hyperpigmented site of the face; Wada's sweating test of the face 10^{-6} in both hyper- and de-pigmented sites. Course: After the above-mentioned treatment for six months the depigmented spots recovered the normal skin color. After eight months' treatment, she looks a normal but darkcomplexioned Japanese.

Case 8. S. S., a woman, aged 25, had had chloasma-like pigmentation on the face treated by a physician with monobenzylether of hydroquinone since about a year before. After three months' treatment, numerous round depig-

mented spots, up to 1 cm in diameter, were present symmetrically on the cheeks and around the eyes. The treatment was continued with no improvement but rather with aggravation. She first came to our clinic after a year's application of the medicine, showing a characteristic "reticulated type" of leucomelanodermia on the face. Blood pressure 110–50 mm Hg; Aschner's phenomenon, positive; basal metabolic rate +15%.

Case 9. K. Y., a housewife, aged 43, applied a cosmetic containing monobenzylether of hydroquinone for protecting from sunburn in sea-bathing on July 20 1956. On the following morning, she noticed severe redness and swelling on her face. The inflammation reduced after about a week without any treatment, and diffuse pigmentation, dark-brown to brownish-slate in color, was present on the face, especially the forehead, the cheeks and the chin. This pigmentation gradually became darker in color and numerous rice-grain-sized depigmented spots were present on the cheeks. She was first seen in our clinic in September 1956, showing a "reticulated type" of leucomelanodermia. Aschner's phenomenon, positive; Thorn's test −72%.

Case 10. H. Y., a housewife, aged 46, who had chloasma of several years' duration on the face, was treated by monobenzylether of hydroquinone for two months with some improvement. Since then she applied a cosmetic containing monobenzylether of hydroquinone. During the first month, she had two fits of erythema and swelling on the face. Subsequently no such side-effect was noticed. In July 1956, when the cosmetic had already been applied for about three months, she had a fit of acute dermatitis on the face after sea-bathing. The inflammation recovered but pigmentation and depigmentation were present on the cheeks and forehead. Application of the same cosmetic was continued for about a month with no improvement but rather with aggravation. She came to our clinic complaining of her severely disfigured face in September. On the forehead, around the eyes, on the cheeks and the preauricular regions was found diffuse dark browish pigmentation mingled with small depigmented spots. Besides, many round or irregular-shaped depigmented spots, 1 to 5 cm in diameter, were present on the chin, the neck and upper regions of both chest and back. These depigmented spots had a hyperpigmented border just as in vitiligo. This case may be considered to be a combination of "depigmented" and "reticulated" types. Laboratory findings: Blood pressure 115–78 mm Hg; Aschner's phenomenon, negative; basal metabolic rate −2%; Thorn's test (epinephrine) −62%; serum sodium 132 mE/l; serum potassium 5.0 mE/l; potassium tolerance test 42% (abnormal); erythrocyte count 3,800,000, leucocyte count 5,400 (polymorphonuclear 57%, lymphocytes 38%, monocytes 4%, eosinophils 1%); blood sedimentation rate 11 mm (1 hour); Takata serum reaction, positive; histamine scratch test, 35 seconds in both hyper- and de-pigmented sites of the sideneck, 105 seconds in both hyper- and de-pigmented sites of the face, 90 seconds in a normal site of the forearm; Wada's sweating test, 10^{-7} in both hyper- and de-pigmented sites of the face, 10^{-9} in a normal site of the forearm. Course: The above-mentioned treatment combined with some liver-protecting medicines such as glucuronic acid carried on for about six months with some improvement. At the time, laboratory findings

revealed that serum sodium was 144.2 mE/l; serum potassium 4.0 mE/l; potassium tolerance test +32.5% (normal).

Case 11. T. S., a housewife, aged 27, with freckles on the face, had applied a cosmetic containing monobenzylether of hydroquinone. After about three months' application, diffuse, dark brownish pigmentation mingled with many small depigmented spots was present on the cheeks and sideneck, and round or irregularshaped depigmented area, 1 to 5 cm in diameter, appeared on the upper regions of the chest and back. She applied the cosmetic on these regions with no improvement but rather with aggravation. After three months from the onset of these symptoms, she was first seen in our clinic. Symptoms of " reticulated type " of leucomelanodermia were found on the face especially on the cheeks (Fig. 29) and those of " depigmented type " were present on the upper regions of the chest and back. This case seems to be a " combined type ". Laboratory findings: Blood pressure 124–86 mm Hg; Aschner's phenomenon, negative; basal metablic rate +2%; Thorn's test (epinephrine) −50%; serum sodium 134 mE/l; serum potassium 4.5 mE/l; potassium tolerance test +55.5% (abnormal); erythrocyte count 3,400,000, leucocyte count 4,000 (polymorphonuclear 53%, lymphocytes 37%, monocytes 6.0%, eosinophils 4%); blood sedimentation rate 8 mm (1 hour); Takata serum reaction, positive; histamine scratch test, 90 seconds in both hyper- and de-pigmented sites of the upper chest. Course: The above-mentioned treatment was carried on for four months with slight improvement.

Case 12. C. T., a housewife, aged 43, had applied a cosmetic containing monobenzylether of hydroquinone since August 1955. In September, she found her face hyperpigmented. She had given up applying the cosmetic since October, but her face became more hyperpigmented. In December 1956, she was first seen in our clinic, showing diffuse, slaty brownish pigmentation on the face, especially on the temporal, preauricular and mental regions. But no signs of inflammation nor of atrophy could be found. Moreover, the most prominent fact was that no depigmented area could be found in any region of the body. No dermatologist could have differentiated the case from Riehl's melanosis, unless he had a great deal of knowledge about the symptoms of leucomelanodermia due to monobenzylether of hydroquinone and known the fact that she had applied the cosmetic containing the compound. This case was diagnosed as a " hyperpigmented type " of leucomelanodermia. The diagnosis was based on the view that, if she applied the cosmetic continuously, there must appear depigmentation as in a " reticulated " or " depigmented type ". Laboratory findings: Blood pressure 140–84 mm Hg; Aschner's phenomenon, negative; basal metablic rate +7%; serum sodium 136 mE/l; serum potassium 4.0 mE/l; potassium tolerance test +55% (abnormal).

Comment

The clinical and laboratory findings of the above-mentioned cases were summarized in Tables XXIII and XXIV. Histological findings were shown in Table XXV.

TABLE XXIII

Clinical Findings

M: medical application by a physician, C: cosmetical application by the patient, depig: depigmented type, reticul: reticulated type, hyperpig: hyperpigmented type.

Case No.	Age (yrs.)	Reason of application	Medical or cosmetical use	Duration of use (months)	Time from first use to onset (months)	Interval between examination and onset (months)	Type of leucomelanodermia
1	33	chloasma	M	6	3	3	depig
2	30	(dark-complexioned)	C	5	3	4	depig
3	41	chloasma?	C	16	5	11	depig
4	27	chloasma (pregnancy)	C	30	28	4	depig
5	35	freckles	C	12	8	4	reticul
6	31	pigmentation after trauma	M	12	6	7	reticul
7	22	freckless	M	10	8	2	reticul
8	25	chloasma?	M	12	3	9	reticul
9	43		C	1 day?	10 days?	2	reticul
10	46	chloasma	C	6	5	1	combined
11	27	freckles	C	6	3	3	combined
12	43		C	2	1	1	hyperpig

Clinical Findings: The 12 cases were females, 4 being in the twenties, thirties and fourties, respectively. As for the methods of applying monobenzylether of hydroquinone, only 4 patients had been given the medicine by physicians, and 8 patients had used cosmetics containing it for themselves. The average age of the former 4 patients was 27.8 years and that of the latter 8 patients was 36.5 years. This difference in age between the two groups seems to show that older women had habitually used this kind of cosmetics in Japan. The reasons of their application of the medicine were reproted to be chloasma with 5 cases, freckles with 3 cases, post-traumatic pigmentation with 1 case, and with the remaining 3 cases, a wish to become fair-complexioned or to prevent sunburn. The longest period of applying the medecine was 2 years and a half, and the shortest was 2 months. (A patient described as Case 9 said she had applied the cosmetic only once, but whether it was only once is very doubtful.) Most patients had begun to show the above-described symptoms within 3 to 6 months after the first application, but some patients within a month, while another showed no symptoms until after 28 months. A prominent fact is that the application of the same cosmetic (or medicine) had been continued even after the onset of symptoms as mentioned above. Thus the patients were first seen in our clinic after a long time had been wasted

TABLE XXIV
Laboratory Findings

Case No.	Blood-pressure (mm Hg)	Aschner' phenomenon	Basal metabolic rate (%)	Thorn's test (−%)	Serum		
					Na (mE/l)	K (mE/l)	K tolerance test(+%)
1	128-82	−	+17	85	142.4	4.75	40.0
2	120-70	+	+18	67	142.0	4.6	47.8
3	110-60	+	+14	84.6	139.0	4.6	41.3
4	130-70	−	+21	25	144.0	4.4	56.8
5	133-76	+	+14	67	144.0 153.0*	4.1 3.0*	92.8
6	120-80	+	+13	50	148.0 137.0*	4.2 4.6*	27.2 24.4*
7	132-70	−	+28	72	144.0 141.0*	4.1 4.6*	47.8 39.1*
8	100-50	+	+15				
9		+		72			
10	115-78	−	− 2	62	132.0 144.2†	5.0 4.0†	42.0 32.5†
11	124-86	−	+ 2	50	134.0	4.5	55.5
12	140-84	−	+ 7		136.0	4.0	55.0

* After recovery. † After six months' treatment.

TABLE XXV
Histological Findings

Case No.	10			11		
Regions	Hyper-pigmented	Border	Depig-mented	Hyper-pigmented	Border	Depig-mented
Hyperkeratosis	+	+	+	+	+	+
Thinning of st. Malpighii	+	+	+	++	++	++
Degeneration						
Prickle cells	−	+	+	−	−	−
Basal cells	−	+	+	−	−	+
Melanin						
St. Malpighii	++	−	−	++	−	−
St. basale	++	+	−	++	+	−
(Dopa reaction)	++	+	−	++	−	−
Chromatophores	++	+	+	+++	+++	+++
Inflammatory infiltrate in the dermis	−	−	+	−	−	−
Degeneration in the connective tissue	+	+	+	−	−	−

since the onset. For instance, Case 3 had used the same cosmetic for about a year after the onset, until at last she came to our clinic. The present author classified the cases in four types: 1) depigmented type (4 cases), 2) reticulated type (5 cases), 3) combined type (2 cases), 4) hyperpigmented type (1 case). The development of symptoms can be summarized as follows: In depigmented type, there appear depigmented spots with hyperpigmented borders in the same localizations, presenting the same findings as in vitiligo. In reticulated type, on the contrary, a diffuse hyperpigmentation is noticed firstly and close observation reveals the presence of small depigmented spots reticulately arranged in the lesion. To compare these two types: 1) the symptoms appear earlier in depigmented type than in reticulated type (Cases 4 and 9 were exceptions); 2) most patients with depigmented type used the cosmetics while most with reticulated type were applied the medicine by physicians; 3) reticulated type could be improved easier by the above-mentioned treatment than depigmented type.

Laboratory Findings: As Table XXIII shows, most patients showed normal blood pressure but a rather increased basal metabolic rate. One half of the cases presented positive Aschner's phenomenon. All cases but one (Case 4) of the 10 cases examined presented a normal value in Thorn's test. The serum sodium and potassium contents lay within the normal range. However, the results of potassium tolerance tests (p. 99) showed abnormally high values in all cases but one (Case 6).

Considerations on Pathogenesis: A hundred cases of various hyperpigmented dermatoses were treated with monobenzylether of hydroquinone in our clinic[11]. Only 4 cases of them presented abnormally depigmented spots, but they were not so severe and easily healed by proper treatment. We know that the cosmetics contining monobenzylether of hydroquinone had been best sellers and been used habitually by many women in Japan. Therefore, it is clear that the cosmetics brought on the above-mentioned symptoms only in a few cases out of many habitual cosmetic users. Thus, it becomes a subject of discussion what kind of women had suffered from such leucomelanodermia.

Lerner and Fitzpatrick described, "It is an interesting phenomenon that, whereas in white patients normal skin pigmentation is not affected, in Negroes not only the abnormally pigmented areas disappear but also the pigment of normal skin. Hence, there is a difference in the total net effect of local application of monobenzylether of hydroquinone on Negro and on White skin. Some unknown balance between melanin formation and melanin depletion may operate to explain this phenomenon." The patients we examined were Japanese, of the yellow race, whose skin color presents a middle tone between in the white and black races. His-

tological and histochemical examinations revealed that monobenzylether of hydroquinone applied on the patients could bring on a complete depigmentation. While incompletely depigmented lesions showed only a slightly positive dopa reaction in melanocytes and a decrease in number of melanin granules in the basal cells, completely depigmented lesions showed a negative dopa reaction and no melanin granules. These findings make it clear that in the above patients normal skin pigmentation had been affected as in Negroes. Moreover, abnormal hyperpigmentation appeared simultaneously in the patients, and the hyperpigmented sites presented a strong positive dopa reaction in the melanocytes, distinct increase in number melanin granules in the epidermis, and chromatophores that increased in number and were distributed in depigmented bordering zone. Thus, it is a very interesting phenomenon that the yellow race presents a special skin manifestation by the use of monobenzylether of hydroquinone, different from that in either the white or the black race.

The most prominent fact in the results of laboratory examinations is that all cases but one showed abnormally high values in the potassium tolerance tests. The results of the tests in various dermatoses were described and discussed in the previous chapter (p. 99). In hyperpigmented dermatoses, Addison's disease, the present disease, chloasma, xeroderma pigmentosum and Riehl's melanosis presented abnormally high values, but freckels, nevus pigmentosus and nevus Ota showed a normal value. And in depigmented dermatoses, albinism and vitiligo presented abnormally high values and postinflammatory depigmentation showed normal. The abnormal result in this tolerance test is presumed to be based on the hypofunction of the adrenal, especially on the disturbance in mineralcorticoid metablism. Leschi[12], who studied this problem by a similar method to those of Zwemer and of us, concluded that adrenal functions in Negroes show a lower physiological range of that in Whites. From these facts, the adrenal function seems to play a great role in the pathogenesis of the above-described symptoms.

We have known the presence of " pigment labile disposition." (For instance, a patient with vitiligo is much easier depigmented by " Guanofuracin (p. 76) " than normal person. We have utilized this phenomenon in the cosmetical treatment of vitiligo.) However, the word of " disposition " has been first used and investigated after the appearance of lesions such as depigmentation, which suggests the existence of " disposition." In other words, it is not always easy to find the " disposition " in normal individuals. It is a clear fact that above-mentioned symptoms were present in a rather few women in contrast with the crowds of habitual users of the cosmetics. However, is it possible to forecast in each case whether the symptoms will occur or not? The results of examinations in

those reported cases and the descriptions by Leschi suggest the usefulness of the potassium tolerance test for the forecast. And we must pay attention to the fact that hyperpigmented dermatoses such as chloasma and Riehl's melanosis, which present an abnormal result in the tolerance test, are indications of the treatment by monobenzylether of hydroquinone.

The most important problem remains unsolved: What is the mechanism of action of monobenzylether of hydroquinone? In spite of the earnest studies by Fitzpatrick and his collaborators[13], the entire mechanism has not been solved. Yoshida and Togashi studied on the problem from the histochemical points and agreed with the hypothesis that monobenzylether of hydroquinone has no action on melanocytes or the tyrosine-tyrosinase reaction, but it may be split to form hydroquinone, which in turn inhibits melanin formation (Denton *et al.*). But, the problem why leucomelanodermia occurs only in the yellow race can not be entirely explained. Only a further progress in the study of human melanogenesis will throw a light on it.

SUMMARY

Twelve cases of leucomelanodermia due to monobenzylether of hydroquinone were reported. The clinical, laboratory and histological findings were summarized in Tables XXIII, XXIV and XXV. The symptoms are of a special kind which appears in relatively few people out of many habitual users of this medicine in Japan and that has never been found in the white or black race. An adrenal hypofuction, which was found in almost all patients by serum potassium tolerance test, is presumed to play a role in an occurrence of those characteristic figures. However, the pathogenesis remains partly unsolved because a mechanism of action of monobenzylether of hydroquinone on melanin formation has not been solved.

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Fig. 28. Case 2, depigmented type.

Fig. 29. Case 11, combined type, showing reticulated lesion.

Fig. 30. Case 3, depigmented type.

Fig. 31. Case 3, after five months' therapy.

XIII. Guanofuracin-Leucoderma

Since the introduction of nitrofurans into chemotherapy by Dodd *et al.* in 1944, many derivatives of nitrofurans have been synthesized and tested for antibacterial activities. Among the side effects caused by these compounds, the most interesting are the atrophy of seminal tubules and depigmentation of the skin.

The former was first noticed by Prior and Fergusson and extensively studied by Paul and collaborators[1-3]. It was found that Furacin inhibited the citrate formation from pyruvate in participation of acetyl Co-A. The latter was observed in Japan after the topical application of Guanofuracin and has become a most interesting dermatological problem. Guanofuracin in the trade name for 5-nitro-2-furfurylidene aminoguanidine hydrochloride. This compound has high solubility in water. Takano applied this for the topical treatment of conjunctivitis and blepharitis in the forms of aqueous solution or ointment, and obtained excellent therapeutic results in most of the cases. Quite unexpectedly, one to eight months after the cessation of the therapy, there occurred depigmentation of the eyelid and eyelash in about twenty per cent of the cases (Fig. 32). Moreover, in some cases depigmentation was noticed at distant areas of the skin. In the following two years many papers on this "Guanofuracin-leucoderma" appeared in both dermatological and ophthalmological literatures. The entire problem was reviewed by Iijima[4].

The authors have investigated the effects of Guanofuracin on factors

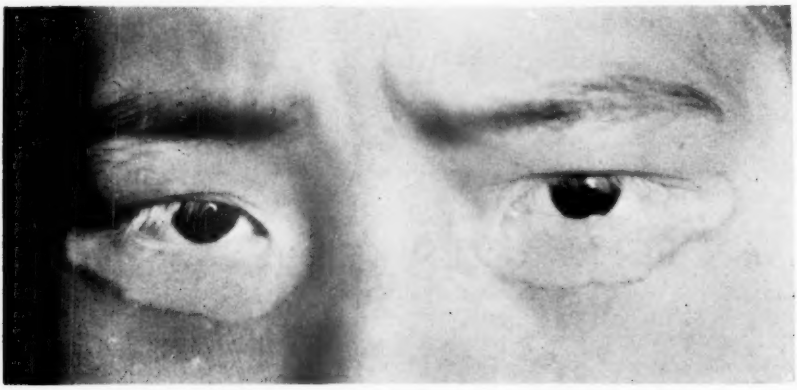


Fig. 32. Guanofuracin-Leucoderma.

participating in the melanin production. Some of the results are given in the following.

The Effect of Guanofuracin on the Activity of Plant Tyrosinase

Experiments and Results

The enzyme was prepared from potato according to Shaaf's[5] method. It was diluted to 2.5 times immediately before use.

0.5 ml of following phenolic derivatives were used as substrate: phenol (10^{-2} M), *p*-cresol ($^1/_3 \times 10^{-2}$ M), tyrosine (suspension), catechol ($1/2 \times 10^{-2}$ M) and dopa ($1/2 \times 10^{-2}$ M).

The activity was determined manometrically using Warburg's apparatus. To the main chamber of the vessel were added 1 ml of 1/15 M phosphate buffer (pH 7.4), 0.5 ml of diluted enzyme solution, water and Guanofuracin to make the total of 3.5 ml. The center well contained 0.25 ml of 40% KOH. After 15 to 20 minutes equilibrium at 37°C, the substrate was added from the side arm. The oxygen consumption was followed every minute up to ten minutes in cases of *p*-cresol and catechol, at five minutes interval up to 120 minutes in cases of other substrates.

The oxygen uptake in initial five minutes period in cases of *p*-cresol and catechol, and that in initial 30 minutes for other substrates were taken for the measure of activity. As shown in Figs. 33–37, Guanofuracin inhibited the activity of potato tyrosinase in the present experimental conditions. This inhibition was reversed by the addition of copper sulfate.

Fig. 33

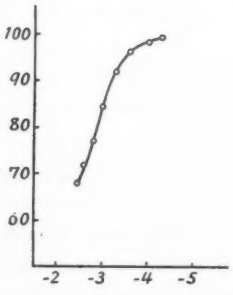


Fig. 34

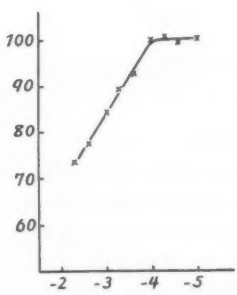


Fig. 35

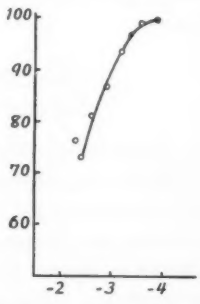


Fig. 33. Phenol-Tyrosinase. Ordinate, per cent activity; abscissa, logarithm of the final molar concentration of Guanofuracin. The results are expressed in the same way thoughout Figs. 33 to 37.

Fig. 34. Tyrosine-Tyrosinase.

Fig. 35. *p*-Cresol-Tyrosinase.

Fig. 36

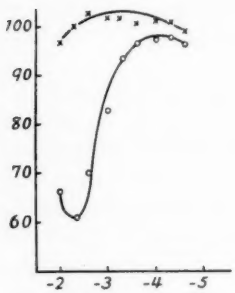


Fig. 37

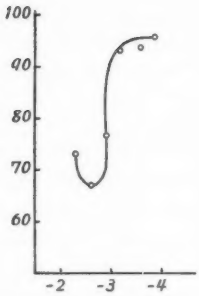


Fig. 38

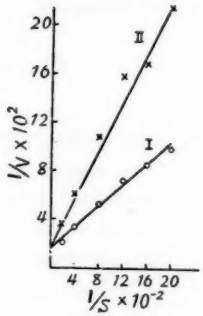


Fig. 36. Dopa-Tyrosinase. ×—× Calculated from the oxygen uptake after 30 minutes.

Fig. 37. Catechol-Tyrosinase.

Fig. 38. Ordinate, negative of the maximal reaction velocity (maximal oxygen uptake during 20 minutes period in the linear portion of reaction curves), abscissa, negative of phenol concentration.

Curve I, 1/564 M Guanofuracin ; curve II, no Guanofuracin.

In case of phenol oxidation, the negatives of the reaction velocities at various substrate concentration in the presence of 1/564 M (50% inhibition) of Guanofuracin were plotted against the negatives of substrate concentrations. The intercept of the resulting line at the ordinate coinsided with that in the absence of Guanofuracin. Thus it was obvious that Guanofuracin is the competetive inhibitor of tyrosinase (Fig. 38).

In other experiment it was demonstrated that Guanofuracin interacted with cupric sulfate by analyzing the ultraviolet absorption spectra (Fig. 39).

The Effects of Chemicals Structurally Related to Guanofuracin on the Activity of Plant Tyrosinase

Experiments and Results

Eight chemicals structurally related to Guanofuracin as shown in Fig. 40 were tested for the effects on potato tyrosinase. The enzyme preparation was similar to that in the previous section except that it was somewhat more purified. 0.5 ml of 2×10^{-2} M *p*-cresol were used for substrate. The substances other than guanidine and aminoguanidine were dissolved in propylene glycol. The same amount of propylene glycol was added to the control flasks in place of water because reaction velocity was low in the presence of propylene glycol.

Guanidine and aminoguanidine had no effects on the activity. The

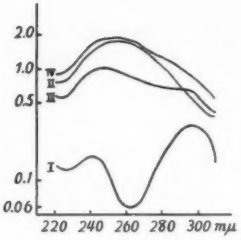


Fig. 39. The absorption was measured with Hitachi autorecording spectrophotometer. Ordinate, extinction; abscissa, wave length.

Curve I, 10^{-4} M $CuSO_4$; curve II, 10^{-4} M Guanofuracin, curve III, mixture of 10^{-4} M $CuSO_4$ and same amount of equimolar Guanofuracin; curve IV, obtained by summing up curves I and II.

induction period was shortened in the presence of these two. Among other nitrofuran derivatives the most effective was the one with thiohydantoin ring in the side chain (Table XXVI & Fig. 40).

TABLE XXVI

Compound no.*	Solvent	Final concentration	Inhibition† (%)
1	Propylene glycol	1/2×10 M	26.0
2	"	"	27.3
3	"	Half saturated	17.0
4	"	"	56.0
5	"	"	12.0
6	"	"	−
7	"	"	−
8	Distilled water	1/2×10 M	−‡
9	"	"	−‡

* Explained in Fig. 40. † Calculated from the oxygen consumption during initial 30 minutes period. ‡ The induction period was shortened.

The Effect of Guanofuracin on Melanoma Enzyme

Experiments and Results

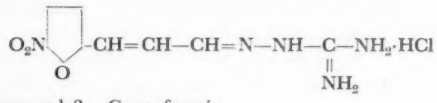
The material used was the primary malignant melanoma of the leg in a 59 years old female patient. 2.4 g (fresh weight) of the melanoma tissue was ground with 4.8 ml of 1/15 M phosphate buffer pH 7.4, centrifuged at 3000 r.p.m. for five minutes and the supernatant was used. The activity was measured in Warburg's apparatus using 1 mg of dopa as substrate. Because of the paucity of the material, the determination was made only at the final concentration of 0.03% of Guanofuracin.

In flasks with Guanofuracin, the oxygen uptake in 15 minutes was 90% of the controls.

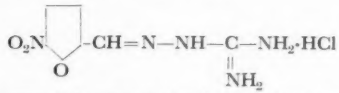
The enzyme preparation showed no activity toward tyrosine.

Fig. 40

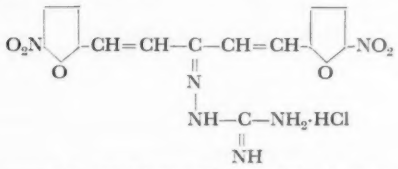
Compound 1. 2-(5-Nitro) furylpropenylidene-aminoguanidine hydrochloride.



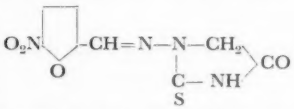
Compound 2. Guanofuracin



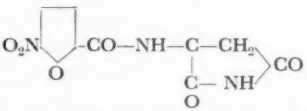
Compound 3. Bis-(5-nitrofurfurylidene)—acetoguanylhydrazon



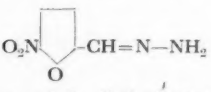
Compound 4. N-(5′-nitro-2′-furfurylidene)—1-amino-2-thiohydantoin



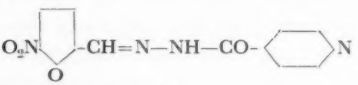
Compound 5. 1-(5′-nitro-2′-furoyl)—Aminohydantoin



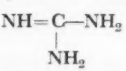
Compound 6. 2-(5-nitro)—furfurylen hydrazine



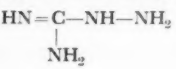
Compound 7. N-(5-nitro)—furfuryden-N′-isonicotinyl hydrazine



Compound 8. Guanidine



Compound 9. Aminoguanidine



The Effect of Guanofuracin on the Dopa Reaction of Human Skin

Experiments and Results

The slide glass method of Iijima and Watanabe[6] was employed.

In some of the experiments the reactions were run in the presence of $1/6\times10^{-2}$ to $1/6\times10^{-9}$ M of Guanofuracin. In other experiments frozen sections were incubated in the solutions of Guanofuracin at 4°C or at 37°C before the reaction.

Contrary to expectation, the presence of Guanofuracin in the reaction mixture did not affect the result of the reaction. In some instances the reaction was somewhat accelerated, especially in the higher concentration ranges. In specimens treated with guanofuracin before the reaction, the results were not uniform.

The Effect of Guanofuracin on the Red Cell GSH

Experiments and Results

In six rabbits 1% aqueous solution of Guanofuracin was injected intravenously five times a week in the amount of 1 ml per kg body weight. On the sixth day blood samples were withdrawn and red cell GSH was determined amperometrically with the method described by Bidmead and Watson[7]. The red cell count, hemoglobin content and hematocrit were determined simultaneously.

As shown in Table XXVII, red cell GSH tended to be high after treatment although statistically insignificant. Moderate anemia developed in 4 of the animals.

TABLE XXVII

Red Cell SH after Guanofuracin Injections

Rabbits no.	Red cell SH (Mg glutathione/100 ml packed red cells)			
	Before injection	After injections for		
		1 week	2 weeks	3 weeks
1	114.8	123.5	136.7	129.4
3	136.2	142.3	119.1	104.9
4	91.1	87.9	113.9	105.1
5	100.3	112.3	117.9	94.4
6	77.2	99.6	104.2	110.7
7	81.9	98.2	104.5	98.8
Mean±s.d.	100.3±22.12	109.0±22.12	116.1±11.98*	107.2±12.24

* $0.1<P<0.2$; $t=1.404$.

The Effect of Guanofuracin on Serum Tyrosine, Serum Copper and Serum Oxidase

Experiments and Results

Five rabbits were treated in the same way as in the previous section. Serum tyrosine was determined with nitrosonaphthol method of Udenfriend and Copper[8]. Serum copper was determined with modified Gubler *et al's*[9] method. The final colored complex was extracted with isoamyl-alcohol. Serum oxidase was determined with the method of Hornykiewicz and Niebauer[10] with slight modification.

No changes were noticed in serum tyrosine and serum copper after the injection of Guanofuracin. The slight increase in the activity of serum oxidase was of high statistical improbability (Table XXVIII).

TABLE XXVIII

Serum Copper, Serum Tyrosine and Serum Oxidase after Guanofuracin Injections

	No. of determ.	Before injection	After injections for		
			1 week	2 weeks	3 weeks
Serum copper* (range)	5	79±5.8 (73–85)	76±16.2 (63–95)	77±8.8 (63–87)	75±7.0 (63–81)
Serum tyrosine† (range)	5	2.1±0.21 (1.8–2.4)	2.1±0.25 (1.7–2.3)	2.1±0.25 (1.8–2.3)	1.9±0.29 (1.5–2.3)
Serum oxidase‡ (range)	5	20.8±3.18 (17.0–23.5)	27.3±14.55 (13.2–47.2)	27.0±7.02§ (21.0–36.8)	24.0±4.14 (19.2–30.0)

* μg/dl. † mg/dl. ‡ μl O/hr. § four determinations.

Comment

From the results of clinical observations there hardly remains doubt that Guanofuracin actually gives rise to depigmentation of the skin without foregoing inflammatory changes. Moreover, it is possible to produce leucoderma experimentally by intracutaneous injections or iontophoresis of Guanofuracin. In leucodermatous skin the dopa reaction is negative. There is no morphological change in the ordinary structures of the epidermis and the corium other than absence of melanin. It may be interesting to examine whether the achromic melanocytes show morphological change or not.

Concerning the pathogenesis of Guanofuracin-leucoderma, the opinions of investigators are not uniform. None of the known properties of nitrofurans as well as aminoguanidine can be made responsible for the pathogenesis of the depigmentation.

Some authors maintained that Guanofuracin-leucoderma was caused

by some change of the nervous system. This hypothesis is chiefly based on the observation that induced sweating was diminished or abolished in the leucodermatous areas. According to Yamada[12], the absence in sweating was noticed in experimental Guanofuracin-leucoderma as well, and preceded the occurrence of actual depigmentation. He also stated that although the decrease in sweating was also observed in vitiligo patches, the absence of induced sweating was specific to Guanofuracin-leucoderma. It is interesting to note that the same author found no histological changes in ecrine sweat glands. The cholinesterase activity was not examined much to the regret. There was no consistent change in the morphology of nerve fibrils stained with silver impregnation methods. Impairment in the sensibility in the depigmented skin was reported in exceptional cases.

The relationship between nervous system and melanin production has been extensively investigated by many others. However, the nervous control of melanin production in human is not yet clear. It is utterly unknown what change in what part of the entire nervous system gives rise to depigmentation of the skin. So, unless substantiated by more concrete findings and theories, the present authors can not hold to the nervous theory of Guanofuracin-leucoderma.

While on the other hand, the results of manometric experiments clearly demonstrated that Guanofuracin inhibited the activity of potato tyrosinase. Similar results were obtained also by Ishida and Sano. Moreover, it was shown that the Guanofuracin was a competetive inhibitor and that it combined with cupric sulfate in vitro. This is an important finding, because most substances which inhibit plant tyrosinase by combining with copper also inhibit mammalian tyrosinase. It may be concluded that Guanofuracin-leucoderma is due to the inhibition of tyrosinase.

This conception, however, is not always confirmed by experimental results using human enzyme preparations. The effect of Guanofuracin on human melanoma enzyme could not be thoroughly investigated because of the paucity of the materials. In one experiment, the oxidation of dopa was inhibited by 10%. Whether such slight inhibition is of any significance awaits further confirmation. Much more complicated was the effect of Guanofuracin on the dopa reaction. Although Yoshida[11] reported that the reaction turned to negative after 5 intracutaneous injections of 0.5% solution, the results of most investigators, including present ones, did not reveal any inhibitory effects on the dopa reaction.

The negative results using human enzyme preparation may look incompatible with those obtained using plant enzymes. However, it is the opinion of the author that this contradiction is only apparent and can be explained by following considerations.

First, it is shown that Guanofuracin competes with the substrates. Consequently, in the presence of substrates in high concentrations as in dopa reaction, the inhibition is reduced to minimum. The second, and which is the most plausible, is that mammalian enzyme is not in the state of true solution but is always attached to particulate matters of the cells. Guanofuracin interacts not only with the copper of the tyrosinase but also with other groups of cellular constituents. It is a known fact that nitrofurans in general react with SH group. On the other hand, the guanidine moiety of Guanofuracin is a denaturating agent for proteins and can reveal masked SH of proteins. It is possible that such reactions precede the one with copper of the tyrosinase and it takes considerable time for Guanofuracin to react with tyrosinase and inactivate the activity ..... a condition impossible to reproduce in dopa technique. The failure to detect inhibitory action of Guanofuracin with mammalian enzymes might be explained by such circumstances.

It cannot be neglected that injections of Guanofuracin resulted in the increase of red cell SH in animal experiments. Tokunaga found that red cell SH was high in vitiligo. The increase was more prominent in cases with shorter duration of the disease. He suggested that this played an important role in the etiology of vitiligo. Similarly the increase in red cell SH after Guanofuracin injection may play an important role in the pathogenesis of Guanofuracin-leucoderma.

Summary

The effects of Guanofuracin upon factors concerned in melanogenesis were studied.

Guanofuracin inhibited potato tyrosinase competetively. The inhibition was reversed by copper.

It was demonstrated spectrophotometrically that Guanofuracin interacted with cupric sulfate.

The inhibition on the melanoma enzyme was only slight and amounted only to ten per cent. The effect on the dopa reaction of the skin was negative.

It was stressed that the absence of inhibition on human enzyme preparation was only apparent, and several possible explanations were proposed.

In animal experiments, red cell SH increased slightly after Guanofuracin injections, while serum copper, serum tyrosine and serum oxidase showed no consistent variations.

It was proposed that Guanofuracin-leucoderma is caused by inhibition of the activity of tyrosinase. The effects on SH metabolism may play some role in the development of depigmentation.

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XIV. L. Q. Z. in Pigmentary Disturbances

As melanin is formed enzymatically in the skin, the pH change of the skin has a profound influence on the melanin production. Dejust[1], Ropshaw[2] and Cailliau[3] pointed to the importance of dermo-epidermal pH on the melanogenesis. Itô and Nakajo[4] observed that black goldfish gradually turned to red after prolonged injection of hydroquinone. As hydroquinone causes persistent alteration of the alkalization potency of rabbit skin, the inhibition of melanin formation in goldfish was surmised to be due to the same mechanism, *i.e.* the impairment of the alkalizing potency of the skin.

In this paper the authors will report the effects of chemicals, which are known to affect melanin production in vitro and in vivo, on the alkalizing potency of the skin.

Experiments and Methods

The alkalizing potency of the skin was measured by the lackmus wheal reaction of Leszezynski and Falik[5]. The principle of this method is to make two wheals with acid and alkaline lackmus solutions and to measure the time for the wheals to become same in color.

Acid lackmus solution: 0.5 ml of 1% lackmus solution (Merck) were added to 9.5 ml of M/15 phosphate buffer (pH 6.46).

Alkaline lackmus solution: 0.5 ml of lackmus solution were added to 9.5 ml of distilled water. Final pH of the solution was 7.0.

Animal experiment: 0.1 ml of acid solution was injected to the dorsal skin of albino rabbits intracutaneously, immediately followed by the injection of alkaline solution to the adjacent skin area. The time interval in which the colors of the two wheals become indistinguishable macroscopically was measured (L.Q.Z., abbreviation of Lackmusquaddelzeit). The results in control animals agree within the error of two minutes.

The substances to be examined were appropriately dissolved and injected sub- or intracutaneously to the remote area from the site of the L.Q.Z. test. Guanofuracin was exceptionally injected into the ear vein. Thereafter changes of the L.Q.Z. were followed up.

Clinical experiment: The method was essentially the same with the animal experiment except that the test site was the flexor surface of the upper arm.

Results and Discussion

Effects of Chemicals on the L.Q.Z.

1) Parahydroxyphenyl and phenyl compounds. The injection of monobenzylether of hydroquinone (2 ml of 3% solution) as well as hydroquinone caused increase in the L.Q.Z., suggesting that the former is converted to the latter (Denton *et al.*[6]).

The injection of aniline (undiluted, 0.2 ml) and paraphenylenediamine which form complexes with quinones, also resulted in the prolonged L.Q.Z. On the other hand L.Q.Z. shortened after the injection of parabenzoquinone (2.5 ml of 2% solution).

Thus the hydroxyphenyl compounds and phenol derivatives tested affected the alkalizing potency of the skin and the resulting change in the pH of the skin influenced the melanogenesis by lengthening or shortening the induction period of tyrosinase.

2) Organic sulfur compounds. After the injection of BAL (0.5 ml of 10% solution), thiourea (5 ml of 2% solution) and methylthiouracil (5 ml of 2% solution intravenously) the L.Q.Z. was shortened. Benzylchloride (0.1 to 0.3 ml), which has anticysteinic action, caused prolongation of the L.Q.Z. It is well known that these organic sulfur compounds inhibit the activity of tyrosinase by combining with the copper of the enzyme. It is improbable that the change in the alkalizing potency of the skin is involved in the inhibitory actions of these chemicals.

3) Guanofuracin. Guanofuracin gives rise to depigmentation of eyelashes after topical application. Intravenous injection of guanofuracin (2.5 ml of 1% solution every other day) resulted in the shortening of the L.Q.Z. after initial two injections. However, after 7 injections the L.Q.Z. rather lengthened than the initial value. Iijima and Tokunaga[7] reported that this compound inhibited potato tyrosinase by combining with copper. However, the effect of guanofuracin on the pigment forming system of human skin is still obscure. Thus no definite results were obtained in the present investigation.

L.Q.Z. in Clinical Disorders

The results are summarized in Table XXIX. In Riehl's melanosis, lichen pigmentosus, chloasma and in nevus Ota, the L.Q.Z. was shortened in about one third of cases. It is interesting to note that in Riehl's melanosis and in Ota's nevus, the pigmentary changes occur chiefly in the dermis. In these disorders, at least in some of the cases the pH change and its influence on the oxidation-reduction system of the skin are thought to be the abnormal accumulation of melanin in the skin.

While on the other hand, no significant changes in L.Q.Z. were observed in vitiligo, albinism and in Addison's disease. As the lackmus wheal method reflects predominantly the pH change of the dermis, the disturbance in these diseases characterized by exclusively epidermal pigmentation may not be detected by this method.

TABLE XXIX

Diseases	No. of cases	L. Q. Z. (Minutes) 3	4	5	6	7	8	9	10
Normal controls	45		1	7	8	14	12	2	1
Nevus pigmentosus	13		1 (7.7%)	5	2	4 (92.3%)	1		
Lentigo	13		1 (7.7%)	6	4	(92.3%)	2		
Ephelides	13		2 (15.8%)	6	4	1 (84.2%)			
Nevus Ota	13		4 (30.7%)	1	4	4 (69.3%)			
Riehl's melanosis	10	1	2 (30.0%)	3	2	1 (70.0%)	1		
Lichen pigmentosus	5		1 (20.0%)	2	1	1 (80.0%)			
Postiflammatory pigmentation	8		1 (12.5%)	2	2	3 (87.5%)			
Chloasma	35	2	8 (28.1%)	8	7	5 (69.1%)	4		1 (2.8%)
Addison's disease	7			3	1	1 (100.0%)	2		
Acanthosis nigricans	2				1	(100.0%)	1		
Albinism	1			1					
White spot disease	2			1	1				
Vitiligo	47	2	4 (12.8%)	20	12	5 (84.8%)	3	1	(2.3%)
Harada's disease	1			1					
Postinflammatory depigmentation	1								1

SUMMARY

From the results of above-mentioned observations it was concluded that some chemicals affected melanogenesis by inducing pH changes of the skin. While on the other hand, in clinical disorders, factors other than pH changes also participate in the pathogenesis.

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XV. Peutz's Syndrome

In 1896 Hutchinson[1] reported on 13-year-old identical twin sisters with numerous ephelides on the lips and the oral mucosa, emphasizing the striking similarity in distribution and size between the two girls. In 1921, Peutz[2] received attention for reporting on a case of ephelides on the oral mucosa in combination with polyposis in the nose and the intestine, strongly indicative of heredity. In 1946, Touraine and Couder[3] reported on 30 cases similar to that of Peutz, collected from literature, and one case of his own finding; in 1949, Jeghers *et al.*[4] gave a detailed report on 10 cases under his observation and some more cases found in literature. In 1954, Troxell[5] stated that the number of reported cases, including one by himself, had reached 38. According to Jeghers, "this syndrome consists of two features: distinctive melanin spots of the buccal mucosa and lips—the face and digits may be involved to a variable extent, but the mouth pigmentation is the *sine qua non* of this portion of the syndrome; and polyposis (synonyms are adenomatosis and papillomatosis) of the small intestine. The stomach, colon and rectum may be involved, but the presence of polyps in the small intestine is the constant feature of this portion of the syndrome. And sufficient genealogic data are reported to demonstrate the hereditary nature of the syndrome, which appears to be inherited as a simple mendelian dominant. Sporadic cases also occur." Apart from these, in his treatise "Zur klinisch-ätiologischen Analyse und Systematik kleinfleckiger Pigmentierung, nebst Beschreibung einer besonderen Form von Epheliden" (1928) Siemens[6] pointed out a specific type of freckles which occur in different regions from those of the common freckles, that is, show pigmented spots on such regions of the face as eyelids, mouth and furrows, and on the conjunctivae, oral mucosa, and palms, contending that those famous "Naevi spili der Mundschleimhaut bei Vorhandensein von Naevi spili der Haut in ausgedehnter, ungewöhnlicher Form" were not nevi, either, but ephelides. In 1929 Siemens[7] in *Jadassohn's Handbuch* described this specific type of ephelides under the designation of "Ephelides inversae", stating that the cases reported by Peutz might belong to this type. In Japan, on the other hand, the present author[8] (1951) experienced 2 cases of ephelides-like pigmented spots on the volar surface of the fingers and the toes, the palms and soles, lips and the oral mucosa, and pointing out the similarity to Siemens' ephelides inversae, made a comparative study of them and the cases reported by Tsuru[9] (1936), Ohmori[10] (1942) and Mori[11] (1948) selected from Japa-

nese literature, and those by Siemens and Szántó[12]. Thereafter Moriyasu and Yoshimatsu[13] (1952) reported similar case, but the combination with polyposis was found in none of them. Subsequently, however, Nagasu and Abe[14] (1955) reported for the first time in Japan on the cases of pigmented spots in combination with polyposis, identical with the cases of Peutz; Kitamura *et al.*[15] (1955) recognized polyposis in 2 of the 5 cases of pigmented spots on lips and the palms and soles of their own finding, regarding them as so-called phacomatosis, and Kawamura[16] approved it, stating that those cases might be regarded as nevi of neural crest origin if the polyposis originated in neurinom or other cause in the neural crest; and Yamazaki *et al.*[17] (1956) added 2 cases of Peutz-Jeghers' syndrome or Kitamura's phacomatosis, thus contributing to the unexpected increase of attention to this type of ephelides. Kawamura[18] has repeatedly emphasized the pigmentation on the palms and soles, being centered in crista profunda intermedia, predominantly characteristic of this disease. This finding was first described by Mori (1948), who found an analogy in the pigmented spots on the monkey's palms and soles, which was revised by Kitamura[19] (1956) who thought that the analogy, if any, was very slight because there is some difference between man's such spots and the monkey's. The present author[8] recognized in his own cases that the pigmentation in the palms and soles is centered in crista profunda intermedia, but taking a stand against Mori who takes them to be atavism, and considering this pigmentation as a type between ephelides and nevus spilus, stressed that the designation by Siemens should be followed, if tentatively. On the other side, Kitamura's view to regard this disease as nevus was followed by that of Troxell (1954), who wrote "the 'phakomatoses' at least include the following: ..... (the sixth or last item) Peutz's syndrome of melanoplakia and small intestinal polyposis."

The present author encountered 3 such cases during 1956. They will be reported below.

Case Report

Case 1. Y. K., 31, man. Family history: Father (63) and mother (58) were healthy. They had 8 children, of whom the eldest daughter had died of gastric ulcer (?) at 40, the second daughter (35) was healthy, the third child and the eldest son was the patient; the second son (28), the third son (26), the third daughter (23), the fourth daughter (21) and the fourth son (16) were all healthy. The parents' was no consanguineous marriage. The patient had a daughter (5) and a son (2). His wife had had two stillbirths. History of previous illness: He had received a months' treatment of jaundice at 29. History of present illness: Since around August 1955 he had had fits of colic 2–3 times a week, had 2–3 diarrhoeas daily. Since the middle of October he had had 2–3 fits of stomach ache daily. Since the beginning of November the condition had got

worse; he had bloody excrements and his temperature rose to 39°C and something. He was received in a certain hospital, where his fever was broken under various kinds of treatment for colitis ulcerosa as his case was diagnosed, but he continued to have bloody excrements. In December he was hospitalized in the Internal Department of our Hospital. Findings at the time of hospitalization: He complained chiefly of an oppressing pain in the left lower abdomen. Hemogram: Hb, (Sahli), 40%; R.B.C., 3,600,000; W.B.C., 6,600; neutrophils 58% (I, 5%; II, 25%; III, 23%; IV, 5%); lymphocytes, 34%; monocytes, 5%; eosinophils, 3%. Blood sedimentation rate, 1 mm per hr.; blood syphilis reaction, (−); uric protein and sugar, (−), urobiliongen, (−). Culture of feces for bacilli, (−).

Course: He made hardly any favorable progress even by cortisone and blood transfusion. Meanwhile romanoscopic and x-ray examinations revealed polyposis in the small intestine. In the meantime his case was diagnosed as Peutzs' syndrome at our Dermatological Department on the basis that he had dark brown ephelides-like pigmented spots of half or full rice-grain size on the lips, oral mucosa, palms and soles. Laboratory findings: Total serum protein, 4.82 gr/dl; Albumin, 47.7%; Globulin, 52.3%; α, 16.4%; β, 11.3%; γ, 24.6%; A/G, 0.91. Serum K, 4.76 mE/l; Na, 138.40 mE/l; Potassium tolerance test, +28.13% (normal). Thorn's test, −48%. This patient left hospital before showing favorable progress, but it has been known that he has since ceased to give bloody excrements, recovering his almost perfect health.

Case 2. E. S., 23, man, clerk of a store. Family history: Father (54) had stomach disorder from the age of 25 through 44 or so. Mother (51) was healthy. On the father's side, grandfather had died at 32 and grandmother died of apoplexy at 71. On the mother's side, grandfather was 72, healthy, and grandmother had died at 40. His parents had seven children: daughter, 27; the patient; daughter, 20; son, 18; daughter, 16; daughter, 13; and daughter, 10. They were all healthy. History of previous illness: He had suffered measles and whooping cough at age of 3, and pleuritis and joint rheumatism at 6. He had for about 4 months been suffering from optic disturbance, which had been diagnosed as opacity of the vitreous body. History of present illness: Since childhood he had a weak digestion, had always been feeling saturated and often been constipated. About 3 months before, an oculist who had diagnosed his case as opacity of the vitreous body, advised him to receive a close internal examination. Polyps being demonstrated roentgenographically at our Internal Department, he was admitted into our hospital. His main complaints were feeling of saturity, poor appetite, an oppressive feeling at the stomach, and pyrosis. He had no nausea, vomiting nor hemoptysis, though he gave a positive reaction to occult blood test. He had recently lost approximately 5 kg. Present status: No remarkable changes were found except the pigmented spots on the lips, palms and soles (Figs. 41–43), which were quite identical with the typical ephelides inversae (Figs. 44, 45), and an oppressive feeling in the gastral region. Laboratory tests: Adrenaline test (−); atropine test (−); pilocarpine test (+); Robinson-Power-Kepler's water test (−); total serum protein, 6.86 g/dl; Albumin, 53.88%; α-Globulin,

9.05%; β-Globulin, 9.48%; γ-Globulin, 27.59%; A/G, 1.17. Thorn's test, −42%, 17-ks 11.52 mg/day (normal). Serum Na 138 mE/l, K 4.5 mE/l; Potassium tolerance test, +46.8% (within 30%—normal). Blood Wassermann test (−). Gastric juice: free hydrochloric acid, 10; total acidity, 20; occult blood test (−). X-ray examination demonstrated one polyp in the gastral region and a finding in the small intestine, suggestive of polyposis, though it was not confirmed. Histological findings: A section removed from the palm, showed same findings as those in the cases of ephelides inversae previously reported by us. That is, melanin granules are richest in the basal layer corresponding to crista profunda intermedia (Fig. 46).

Findings in the family: Father with slight ephelides had previously had severe gastroenteric troubles. He had 7 children, of whom the eldest daughter (27) was healthy, the eldest son was the patient, the second daughter (20) had pigmented spots on the oral mucosa and the left sole; serum Na 136 mE/l, K 4.9 mE/l, potassium tolerance test +28.4% (normal), the second son (18) was healthy, the third daughter (16) had pigmented spots on the palm and lips, the fourth (13) and the fifth (10) daughters were all healthy. The parents' was no consanguineous marriage.

Case 3. H. I., 20, woman, student. Family history; Mother, 50, also had pigmented spots identical with those of the patient; had suffered from gastroneteric disturbances 10 years before; had been hospitalized several times for close examinations and told that she had polyposis in the stomach and intestines. Father and elder sister, 26, were healthy. History of previous illness: At age 5–6, ephelides-like pigmented spots had appeared on her face, especially lips. Similar ones had later appreared on the palms and soles, especially on the tips of fingers and toes. Her stomach and intestines had been rather weak, but they had not been with any disorders to be specially mentioned. Histoty of present illness: She had ephelides on the face, brown pigmented spots of a half-rice size on the conjunctiva bulbi of each eye; several tiny, dark-brown, ephelides-like pigmented spots around each nostril; several pigmented spots ranging in size from a point to a half rice on the lips, especially on the lower lip; and similar spots on the oral mucosa, particularly on the buccal mucosa, on the palms and soles, especially on the tips of fingers and toes. Advice was given to continue to visit our hospital for close examination, but she paid no more visit.

Comment

It is not clear whether the 3 cases (man, 17; man, 22; and woman, 31) formerly reported as ephelides inversae by the present author[1] were associated with polyposis at all, because they had not complained of gastroenteric disturbances in particular nor received x-ray examinations. Each one of the present 3 cases, as the former 3, presented specific pigmented spots, similar to those described by Siemens as pertaining to ephelides inversae. Cases 1 and 2, moreover, were clearly accompanied with polyposis; and some of the family members of Case 2 were involved

Fig. 41. Case 2.

Fig. 43. Case 2.

Fig. 44. Patient previously reported as ephelides inversae.

Fig. 42. Case 2.

Fig. 45. Same as Fig. 44.

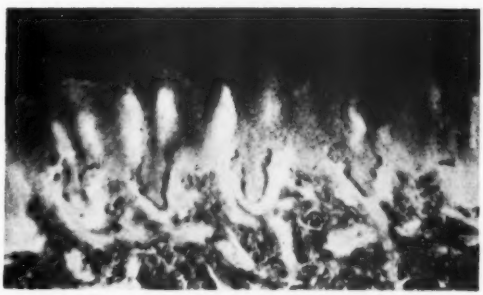

Fig. 46. Biopsy of the skin on the palm of patient previously reported as ephelides inversae, showing melanin granules in the basal layer of crista profunda intermedia. (silver impregnation method)

in the same pigmentation. In Case 3, polyposis was not demonstrated, but it was known that her mother who had presented similar pigmented spots had had her case diagnosed as polyposis. Strictly speaking, however, it was in the large intestine in Case 1 and in the stomach in Case 2 that polypi were evidenced; no positive evidence was given after all of the presence in the small intestine in either case. Therefore it may be doubtful whether these cases are in full accord with the definition given by Jeghers *et al.*[5].

Anyway these 6 cases, including the formerly reported 3, are considered, from the appearance of pigmented spots in particular regions and the very specific histological findings in those appearing especially in the palms, to fall under one and the same category, at least in the respect of pigmented spots. This will open a problem of the relationship between these pigmented spots—or ephelides inversae, as tentatively called in the sense of the word explained in the previous report—and polyposis. Supposing ephelides inversae+polyposis=Peutz's Syndrome in its typical form, can there be (1) a form of Peutz's Syndrome without evident ephelides inversae but with evident polyposis, or (2) an inverse form with evidence of ephelides inversae but with doubtful presence of polyposis? Literary interpretation of the definition by Jeghers *et al.* clearly excludes these so-called incomplete forms from Peutz's Syndrome. In practice, however, the following must be kept in mind. In the form (1), it may be considered also that according to the view of Jeghers who makes pigmentation on the oral mucosa a necessary condition of the syndrome and merely mentions the face, and fingers as sites of possible appearance, the presence on the oral mucosa of pigmented spots, if not characteristic of ephelides inversae, is enough to satisfy the first condition. If so, the fact that we of the yellow race show a fairly high frequency (70% according to Mine[20]) of pigmented spots on the oral mucosa in normal persons must be taken into consideration. Then it is followed by the question whether the pigmented spots on the oral mucosa in Peutz's Syndrome possess any characteristics distinctive from those found in such normal persons. The answer is, for the present, no; there being none of such specific histological findings as in those on the palms. Accordingly, the distinction can only be made, at present, by clinical findings that the pigmented spots in Peutz's Syndrome are far marked than those in normals and that many of them are symmetrical. Thus the satisfactory solution is yet to be found for the problem of the form (1) or the so-called incomplete form of Peutz's syndrome. To turn to the form (2): There have been indeed not a few cases in which pigmentation is observed but the presence of polyposis is not evidenced. No doubt is left when polyposis is evidenced at operation or x-ray examination. But even if no polyposis

is thereby recognized, it will not be logical to declare the absence of polyposis. The determination of the question may be made only autopsy. Thus it follows that with regard to the form (2) or the so-called incomplete form also, the question remains unsolved. Consequently, the solution of the question as to whether there can be the above-described two incomplete forms will require further close observations of a greater number of such cases; all that may well be said at present is that the presence of the above-stated pigmentation indicates the necessity of close examination for suspected polyposis.

As for the views of Troxell[5] and Kitamura[15] who regard this disease as a phacomatosis, they are apt to entail a danger of remaining in the problem of verbal definitions unless the possible relation between these pigmented spots and polyposis is confirmed. Though it can hardly be said that histological investigations have been fully performed of papillom, Jeghers *et al.* consider as much as that "the synonyms of polyposis are adenomatosis and papillomatosis." However, this is not enough to make us believe, as Kawamura,[16] that polyposis is of the neural crest origin. On the other hand, the pigmented spots on the palms present, as pointed out by Mori, recognized by the present author and confirmed by Kawamura in his detailed description, very characteristic histological findings. They show that pigment is centered in crista profunda intermedia. This fact seems to have an important significance from the embroyological point of view also; it might be the key to solve the problem of Peutz's Syndrome. As against the views of Jeghers *et al.* who give prominence to the pigmentation on the oral mucosa, the present author wants to lay stress on the so far neglected importance of the presence of the pigmented spots on the palms—that their histological characteristics also should not be disregarded. So far as the colored races at least are concerned, the evidence and histological examination of pigmented spots on the palms is considered to have a great significance in the diagnosis of Peutz's syndrom.

Summary and Conclusion

Three cases of what appeared to be Peutz's Syndrome have been reported, a comparative discussion made with the 3 cases previously reported by the author as ephelides inversae (Siemens) and those found in literature. The definition of this syndrome by Jeghers *et al.* has been criticized, and the problem as to whether there can be incomplete forms of this disease, such as polyposis without associated pigmentation and the inverse, pigmentation without associated polyposis, has been discussed from the stand-point of practical clinic. It has been stressed upon that the extremely characteristic histological findings in the pigmented spots on the palms and soles have a great significance in the clarification of the

genesis and nature as well as in the practical diagnosis of this disease.

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XVI. Thorn's Test and Potassium Tolerance Test in Pigmentary Diseases

The relation between melanogenesis and the endocrine glands first drew attention in connection with pathology of Addison's disease one century ago. Since then many experimental and clinical researches have been performed on the pituitary, adrenal, gonadal and thyroid glands, and the recent studies by Lerner, Fitzpatrick and Shizume on 'melanocyte-stimulating hormone' (MSH) deserve our respectful attention. But there remains many a chaotic problem yet to be cleared up. The followings are the reports of some of our studies made chiefly on the relation between melanogenesis and the pituitary-adrenocortical system using patients with various pigmentary disorders as subjects.

Thorn's Test

The results of the tests for pituitary-adrenocortical deficiency given after Reant's simplified method using subcutaneous injection of 0.3 cc of a 1/1,000 epinephrine are shown in Table XXX. In some of the patients was combined the original Thorn's method using ACTH.

It is quite natural that the highest percentage of deficiencies was

TABLE XXX

Thorn's Test

	No. of cases	Percentages of abnormal cases
Addison's disease	22	68.2
Riehl's melanosis	42	50.0
Lichen pigmentosus	8	25.0
Postinflammatory pigmentation	18	33.3
Chloasma	67	26.9
Vitiligo	20	50.0
Nevus pigmentosus	11	18.2
Nevus Ota	11	36.4
Albinism	1	0
Nevus depigmentosus	2	0
Ephelides	11	9.1
Xeroderma pigmentosum	6	33.3
Ephelides inversae	2	100.0
Acanthosis nigricans	5	20.0

attributed to Addison's disease, and it was of particular interest that a serious deficiency was revealed by Thorn's test using epinephrine and ACTH in a patient who took Addison's disease-like pigmentation following fracture of the basal bone of the skull. It was also observed that skin pigmentation was alleviated in mild cases by long-term administration of cortisone.

The explanation of the mechanism of pathogenesis of Riehl's melanosis varies with researchers: some advance an external-factor theory describing the disease to abuse of coarse cosmetics, diet, light-sensitization, etc.; others hold an internal-factor theory, and still others an eclectic theory. On the participation of the endocrine functions in the mechanism in particular there have many views published. With the results of Thorn's tests in patients with this disease, Kawagishi, Hayami, Kawajiri, Urahashi and Gondo, Yamura, and Miyagaki and Sakurane have reported that many of those patients showed a lower value of pituitary-adrenocrotical deficiency than the normal decrease range. Low blood pressure, low blood-sugar level and diminished 17-ketosteroid excretion seemed to Spira to indicate disturbed function of the adrenal glands. In our tests, we found the deficiency in about one-half of the total number of cases of this disease. Besides, a part of the tested cases showed a pigmented spot in the mucous membrane of the oral cavity and many of the cases had low blood pressure, while in some cases, the pigmentation faded by continued administration of adrenocortical hormone, adrenalin and vitamin C in combination. We may also mention a woman, 43, who had failed to receive treatment of the incipient Riehl's melanosis for two years until she died of Addison's disease. Such observations lead us to an inference that one of the internal causes of those diseases is the hidden presence of adrenocortical defficiency. The cases of the so-called post-inflammatory pigmentation subjected to Thorn's tests showed the deficiency in more than one-third of the cases, and it seems that the pathogenetic mechanism of this pigmentation is similar to that of Addison's disease.

Chloasma has long been drawing careful attention to its relationship to the endocrine glands, in particular to the gonads. Matthaus noticed hypertrophy of the adrenal glands during pregnancy and called it interrenalismus. Our Thorn's tests proved a deficiency in 27.4% of the patients.

More than half of our cases of vitiligo showed an deficiency by Thorn's test. It has often been reported since Addison's discription of the diesase that approximately 20% of the patients with Addison's disease have vitiligo in complication. Thus it is suspected that the function of the adrenal cortex is a cause of the change in pigmentation of the skin.

The patients with congenital pigmentary anomalies showed a rela-

tively low percentage of deficiencies by epinephrine test, only those with nevus fusco-caeruleus Ota showed a slight deficiency. It is noteworthy however, that the 2 cases of ephelides inversae showed both a high deficiency. The limitation of the sites of the pigmented spots to the lips, the oral cavity, the palms and the soles is a characteristic of this disease which is in common with that of the Addisonian pigmentation. This fact seems to give a hint as to the pathological mechanism of melanogenesis.

Serum Potassium Tolerance Test

Of the small number of researches made so far on the relationship between melanogenesis and electrolyte metablism, only few may be cited here: the experiments carried on by Spoor and Ralli[1] for the fate of melanin in the skin of rats under the conditions of scheduled salt contents in the diet and adrenalectomy, the in vitro experiments by Lea[2] for testing the oxidation of premelanin substances in presence of ascorbic acid using salt diluted media, and the comparative experiments by Pann of the skin colors of eels raised in fresh water and salt water. In 1932, however, Loeb[3] demonstrated a decrease of Na and Cl and an increase of K in the blood of patients with Addison's disease, and laying stress on the unbalance of Na : K, he advocated salt treatment. Subsequently, Sullivan, Marine, Baumann Collazo and Maranon Rynearson, Snell and Hansner, Thaddea, Thorn, Hashimoto, Nakazawa, Tanji, Sakamoto, Saito and Fujimaki observed a decrease in the Na and Cl contents in serum.

Experiments and Results

Method: We administered orally about 6 g potassic salt (220 g potassium citrate, 155 g potassium chloride dissolved in 1,500 cc water; 50 cc per 50 kg bodyweight) to patients with various pigmentary anomalies and measured the K and Na contents in the serum before and after administration, with a flame photometer. The results are shown in Table XXXI in which the changes in the Na content are not included because they were slight.

Results: The results naturally show highly advanced disturbances of mineralcorticoid function in Addison's disease, also revealing fairly advanced disturbances even in chloasma. They correspond to the results of the above-described Thorn's tests in indicating the probable participation of the adrenocortical system in these diseases. Somewhat similar tendencies are also found in Riehl's melanosis and leucomelanodermia due to monobenzylether of hydroquinone (p. 64), the difference between them corresponding to that between the dark-colored group and the fair-colored Japanese group in our experiments for the effects of sunbathing on normal skin which will be described later (p. 102).

TABLE XXXI

Results of Potassium Tolerance Test in Various Pigmentary Diseases

Diseases	No. of cases	Average value (mE/l)		After/Before
		Before	After	
Addison's disease	18	4.85	7.32	1.51
Chloasma	63	4.54	6.39	1.43
Riehl's melanosis	20	4.62	6.56	1.42
Leucomelanodermia due to MBEH	14	4.43	6.55	1.48
Lichen pigmentosus	8	4.76	6.47	1.36
Postinflammatory pigmentation	25	4.57	6.07	1.33
Postinflammatory depigmentation	4	4.40	6.38	1.45
Pigmentatio multiplex maculosa	5	4.65	5.62	1.21
Complete albinism	5	3.63	6.00	*1.82
Vitiligo	67	4.33	6.14	1.42
Ephelides	37	4.51	5.99	1.33
Ephelides inversae	4	4.42	5.65	1.28
Peutz's syndrome	2	4.70	6.48	1.38
Xeroderma pigmentosum	3	4.45	6.59	1.48
Dyschromatosis symmetrica hereditaria	2	5.10	6.80	*1.51
Nevus pigmentosus	45	4.44	5.94	1.34
Lentigines	3	4.80	5.92	1.23
Nevus fusco-caeruleus ophthalmomaxillaris	32	4.52	6.05	1.34
Canities	2	4.15	5.68	1.37
Normal controls	81	4.39	5.84	1.33

* Single determination

That the hyperpigmentary diseases of congenital origin show almost normal values is taken for granted, because they are localized anomalies of inherited characters and have no correlation at all with adrenal functions.

The 5 patients with complete albinism showed K contents in the serum far lower than the lower range in normals, and one (normal in Thorn's test) of them showed an abnormal increase of 81.6% in K content in the serum after administration of pottasic salt. The patients with vitiligo also showed a remarkable increase, suggesting frequent incidences in them of functional insufficiency of the adrenal cortex. These results indeed should have due attention in collation with the view of Leschi that the adrenal functions in Negroes show a lower physiological range of that in Whites and the high occurrence ratio of vitiligo in the colored races (*e.g.* 1 :36 among Negroes according to Garden), a very high ratio

even after due consideration, as suggested by Darier and Lindberg, of a possible difference in detection rate according to a greater of smaller contrast between lesions and skin colors.

Summary

In order to study on the relationship between pituitary-adrenal system and melanogenesis, Thorn's and serum potassium tolerance tests were performed in patients with various pigmentary diseases. The abnormalities were noticed in high rate in the most of acquired hyperpigmentary diseases. Thus, the participation of adrenal cortex in the regulation of skin pigmentation was presumed.

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XVII. Potassium Tolerance Test by Means of Sun-Bathing

The previous chapter dealt with the studies of the relationship between melanogenesis and pituitary-adrenal system, especially mineralcorticoids, in various pigmentary dermatoses. In this chapter will be studied the relationship between the same in normal persons by compairing the results of serum potassium tolerance test before and after sun-bathing, in a fair-complexioned and a dark-complexioned Japanese group.

Experiments and Results

The skin colors of normal Japanese were determined with reference to the color labels of the Japanese Color Research Institute or, in case of difficulty, with the help of the reflectance spectrophotometer, and indicated in terms of luminosity by CIE value (Y), dominant wave length ($\lambda m\mu$) and purity (pe%). Then those subjects were allowed to bath in the sun at a beach in summer: i) 11 normal men and women on 31st of July, 1955; ii) 10 normals at 6th and 8th of August, 1956 (Tables XXXII, XXXIII). Prior to and after sun-bathing, examinations were performed of the changes in K and Na contents in the serum due to potassic salt administration as mentioned in the previous chapter.

1) *Changes in Color Tone:* To estimate the effects of sunbathing, the subjects were roughly divided into two, a fair group with luminosity over 38 and a dark group with luminosity below 38. In the former group the change in color tone, especially the lowering of luminosity was quick, the recovery tended to be also rapid, and the change of dominant wave length formed an acute angle, while in the latter group the lowering and the recovery of luminosity were slow and the process of dominant wave length formed an obtuse angle.

2) *Changes of K and Na Contents in the Serum:* Upon examination of the behavior of mineralcorticoids by measuring the change in the serum K and Na contents following oral administration of 6 g potassic salt, the increase of serum K two hours after administration on the day before sun-bathing was lower in the fair group (26.7%) than in the dark group (37.4%), a result in consonance with that of a similar examination of Whites and Negroes by Leschi. The measurement made on the day after sun-bathing, however, showed an inverted result, the rate of K increase having risen to 47.7% in the fair group as compared with the 40.8% of the other. That is, in the dark group a single sunbath caused hardly

TABLE XXXII

Results of Serum Potassium Tolerance Tests before and One Day after Sun-bathing Performed on July 31, 1955 at a Beach

	Age	Sex	Skin color Luminosity*	Before Sun-bathing		1 day After Sun-bathing	
				Before administration K(mE/l)	After administration increase %	Before administration K(mE/l)	After administration increase %
Dark complexioned	61	M	38	3.6	36	3.7	40
	32	M	25	3.6	39	3.7	41
	28	M	35	4.1	22	3.9	30
	30	M	35	3.7	40	3.5	53
	23	F	36	3.9	40	4.5	40
	Average		34	3.8	37.4	3.9	40.8
Fair complexioned	28	M	45	4.0	20	3.6	46
	28	M	45	3.7	35	3.7	51
	26	M	40	4.1	58	3.7	58
	26	M	38	5.0	13	4.9	55
	34	F	44	4.6	24	4.6	36
	23	F	45	4.8	10	4.5	40
	Average		42.5	4.4	26.7	4.2	47.7

* Average value in both the cheek and extensor surface of the upper arm.

any fluctuation in the rate of K increase, whereas in the fair group it effected a remarkable change.

COMMENT

These results seem to suggest that the sun-light worked as a stronger stress on the adrenal cortex of the fair group because of the higher sensitivity to ultraviolet rays of their fair skin. As described in the previous chapter, most of acquired pigmentary diseases showed abnormal results in Thorn's tests and serum potassium tolerance tests. Considering these results and our present results by means of sun-bathing, an influence of adrenal insufficience on melanogenesis seems to be very important.

In 1952, Leschi[1], who could but anatomically admit—on the bases of the comparative studies by Pearl, Gooch and Freeman of the weight of the adrenal glands in Whites and Negroes and the comparative investigation by Swinyard into the ratios in volume of the whole adrenal glands to the cortex/medulla in both races—the observation that the ratios between the whole adrenal glands and the cortex/medulla are smaller in the black than in the white, but who considered that the determination

TABLE XXXIII

Results of Serum Patassium Tolerance Tests before and One Day after Sun-Bathing Performed on August 5 and 8, 1956 at a Beach

	Age	Sex	Skin color Luminosity	Before Sun-bathing		After Sun-bathing of 2 days	
				Before administration K(mE/l)	After administration increase %	Before administration K(mE/l)	After administration increase %
Dark complexioned	26	F	38	4.5	42.2	4.4	46.3
	26	F	33	4.3	53.5	4.5	25.0
	27	F	35	3.8	23.9	4.5	26.6
	25	F	24	4.8	22.9	4.6	36.9
	29	F	30	4.2	33.5	4.1	53.9
	Average		32.0	4.32	35.2	4.42	37.7
Fair complexioned	21	F	45	4.7	38.3	4.0	65.0
	28	F	41	4.5	46.6	3.9	43.6
	26	F	41	4.0	27.5	4.1	51.2
	25	F	38	4.7	22.5	4.2	42.4
	26	M	41	4.5	40.0	4.3	55.8
	Average		41.2	4.48	34.9	4.1	51.6

of the value of adrenal function naturally required to be endorsed by various physiological researches, tried to know even a part of adrenal function by biochemical procedures. That is, in consideration of the works on the changes of Cl^-, Na^+ and K^+ in the blood corresponding to the functional unbalance of the adrenal glands, Leschi determined such contents in the serum of Whites and Negroes, with the results that the medium, maximum and minimum values of the Cl and Na contents were all higher in Whites than in Negroes but that those of the K content was much higher in Negroes. The determination of the K content in the serum before and after the administration of potassic salt to individuals showing a normal K content revealed that more instances were among the Negroes in which the increase rate was higher. The measurement of the K content in the serum before and after adminstration of DOCA showed that more instances of increase were among the Negroes and more instances of decrease among the Whites. This experiment was undertaken on the basis that disoxycorticosterone, which is a mineralocorticoid compound found in adrenal hormones, influences electrolytic metabolism. An anomaly of electrolytic metabolism such as in Addison's disease was observed in Negroes, and this was explained as showing that the physiological threshold of adrenal functions in Negroes was below that in Whites. This view was

supported by referring to the report by Conn and Mathews that their clinical and autopsy results showed four times as many incidences of Addison's disease in Negroes as in Whites and the observation by Gennes, as cited by Amado, that hardly any of the 300 cases of Addison's disease were blonde. Thus it was inferred by Leschi that the difference in physical constitution and adjustment or adaptability to circumstances between the two races has caused the difference in the threshold value of adrenal functions between them. One may also be referred to the latest (1951 and after) U. S. statistics of mortality showing that the percentage of deaths of adrenal diseases has become higher in the Negroes than in the white. We can not readily agree with the above view, but it seems to give an interesting suggestion on the physiological genetics of the racial difference in color of the skin.

On the high frequency of Mongolian spot and nevus fusco-caeruleus Ota among us of the Yellow race, we must study from this point.

Furthermore, pigmentation of the oral mucous membrane is already known to occur in Addison's disease, vagabond's melanosis and ephelides inversae, but it has also been observed frequently in colored normal individuals (Manson, Reiche[2] and Dummett[3]), though it is very rarely found in the white race (Crouzon and Chateilin). In Japan, it has been studied by Okuguchi[4] and Mine[5], the latter of whom found it in 458 or 32% of the 1,425. It gives almost the same histological findings as Addison's disease, which are mainly accumulations of melanin and positive dopa reactions (Mine and Becker[6]).

Pigmented spots on the palm and the sole are not very uncommon in Japan (Toyama[7] and Kawamura *et al.*[8]—8.6%). Mori and Kawamura have noted that the pigmentation of the palm and sole in ephelides inversae is centered on the crista profunda intermedia, and comparing it with the description of Nakagawa and Adachi[9] that the pigmentation of the monkey's palm is also centered on the crista intermedia, have aroused genealogical interest. Thus the frequency of pigment maculae in the oral cavity, on the lip, the palm and the sole corresponds with that of Mongolian spots in that they are in proportion to the darkness of skin color and are regarded as physiological characters showing an ethnological difference in frequency, though the Mongolian spot is a hyperpigmentation in the corium which fades away with age in contrast to the pigmented macula is a hyperpigmentation in the epidermis which appears at a later age. This is of deep interest if considered in the light of the results of Leschi's ethnological examinations of Cu and K contents in the blood and those of our Thorn's tests and potassium tolerance tests carried out in the examination of adrenal function according to the criteria of pathological and physiological skin-colors. The quantity of pigment distributed in

the skin and the oral cavity varies in somewhat ethnological order, and the threshold value of that part of adrenal function which is concerned with the mechanism of melanin formation appears to show a corresponding difference; in other words, it is inferred that the physiological threshold of adrenal function in the yellow races shows a value standing between those of the white and the Negro races. And the statistical facts that melanoma is aggravated very often in the white race and very rarely in the colored races are observed to make sharp contrasts due to racial differences.

Summary

Serum potassium tolerance tests were made in normal persons before and after sun-bathing, and the results were compared between two groups, that is, a fair-complexioned and a dark-complexioned Japanese group. It was made clear that ultraviolet irradiation gave a stronger stress to fair-complexioned persons than to dark-complexioned persons.

Yellow races have many specific pigmentary manifestations different from those in other races, for instances, Mongolian spot appears in almost one hundred per cent of new borns, nevus fuscocaeruleus Ota is more often found here than in other races, and here again pigmented spots occur also more physiologically in the oral mucous membrane or on the palms and soles. These facts must be studied from the view point of physiological genetics.

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XVIII. 17-Ketosteroids and Sex-Hormones in the Urine

In a preceding chapter we[1] have reported our studies of electrolyte metabolism in patients with various pigmentary diseases by means of Thorn's tests and potassium tolerance tests performed to investigate into the possible relation of melanin formation to the pituitary-adrenal system. In this chapter we will describe the results obtained from our study of the changes in urinary 17-ketosteroids and sex hormones in pregnancy and various pigmentary diseases.

Measurement of Urinary 17-Ketosteroids

In 17-ketosteroids are included a rich variety of steroids, most of which have been pointed out by Selye and Soffer to originate in the adrenal cortex. They are utilized for estimating the extent of dysfunction of the adrenal glands.

Increased pigmentation follows bilateral adrenalectomy in man (Kepler[2], Hall *et al.*[3]) and in other animals (Ralli and Graef[4], Spoor and Ralli[5], Butcher[6]), whereas administration of desoxycorticosterone (Spoor and Ralli, Hamilton[7]), cortisone (Whitaker and Baker[8], Hall *et al.*, and many others) or aldosterone (Mack *et al.*[9], Kekwick and Pawan[10]) to cases of increased pigmentation due to adrenalectomy or adrenal dysfunction causes fading of the pigmentation. Consequently it is supposed that the decrease of adreno-cortical mineralo- and gluco-corticoids will result in an increased pigmentation, and the increase, in a decreased pigmentation. We have made quantitative determinations of 17-ketosteroids (Drekter's method[11]) and the fractions (Suzuki-Adachi's method[12]) in the urine of pregnants and patients with pigmentary diseases of the skin.

Experiments and Results

a. Pregnancy, parturition and puerperium

Pregnancy is accompanied with increased pigmentation in the skin of the nipples, halos, linea fusca and external genitals, which is heightened with the months of pregnancy and begins to fade away gradually during puerperium. To estimate the total amount of 17-ketosteroids excreted in the urine, we measured a total of 52 samples including 6–8 each of the 8 groups of urine taken during the 2nd—10th month of normal pregnancy,

in parturition and on the 3rd and 10th days of puerperium (Fig. 47). The total 17-ketosteroids value does not differ much in the early and the middle stage of pregnancy from that of the normal non-pregnant woman, begins to rise slowly from the 8th month, forms a steep peak at childbirth, and recovers the value of the normal nonpregnant on the 10th day of puerperium.

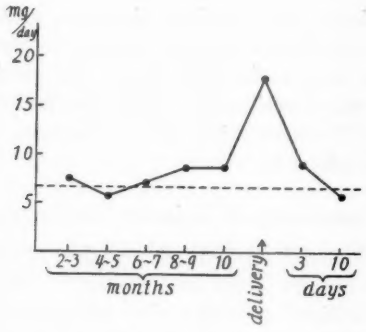

Fig. 47. Values of total urinary 17-ketosteroids in normal pregnancy, parturition and purperium. ----- mean values of normal non-pregnant women.

Furthermore, the 17-ketosteroids extracted from the urine were analyzed into Fractions I-VIII by elution chromatography. The steroids contained in separate fractions and their amounts are shown in Table: Fractions IV and V are the products of androgen metabolism, and Fractions II, VI and VII are the metabolism products of glucocorticoid, adrenosterone, etc. which are the adreno-cortical hormones. The percentages in the fractions are to the values measured in the samples from 5 normal women and 4 normal men (Table XXXIV).

Fractions were measured of the samples taken from 4 cases in the 2nd–3rd months of pregnancy, 3 cases in the 4th–5th months, 3 cases in the 6th–7th months, 3 cases in the 8th–9th months and 4 cases in the 10th month, in 2 of the latter cases measurements being done throughout the whole processes of parturition and puerperium (Fig. 48). Fractions IV (androsterone) and V (etiocholanolone), both orginating in androgen, decrease as compared with other fractions, and increase during puerperium. This result is in contradiction to the known fact that androgen acts in intensifying pigmentation of the skin, as do the female sex-hormones. Hence it is presumed that androgen does not participate in hyperpigmentation during pregnancy.

TABLE XXXIV

Distribution of the 17-Ketosteroids in the Fractions

Figures express percentages of the total 17-ketosteroid excreted in a day

Fraction		Female Excretion 4.5–10.0 mg per day	Male Excretion 5.0–18.0 mg per day
I	Artefacts of hydrolysis: (androst-2-en-17-one, androst-3:5-dien-17-one, 3-chloroandrost-5-en-17-one)	1–20%	2–18%
II and III	*i*-androstenol-6-one-17, the others	10–55%	15–60%
IV and V	androsterone and etiocholanolone	25–50%	28–59%
VI and VII	11-hydroxy-17-ketosteroids	3–25%	4–27%
VIII	unidentified 17-ketosteroids	0–14%	1–13%

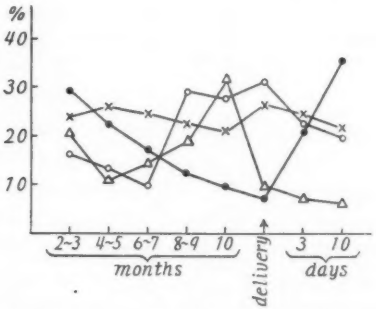

Fig. 48. Changes in urinary 17-ketosteroid fractions in normal pregnancy, parturition and puerperium. △–II. fraction, ×–III., ●–IV. and V., ○–VI. and VII.

b. Pigmentary skin diseases

Table XXXV shows the total amounts of 17-ketosteroids excreted in the urine of the patients during 24 hours.

In Addison's disease, the decrease in the total value of 17-ketosteroids is generally accepted, and in our tests all of the 7 typical cases of the disease showed lowered values. In the measurement of fraction, some cases were without any great changes in any fragment, but others showed a marked decrease particularly in the 11-oxygenated 17-ketosteroids (Fig. 49). A mild case suspected of Addison's disease showed a marked decrease of 11-oxygenated 17-ketosteroids in the determination of fractions, though the total amount of 17-ketosteroids were within the normal range (Fig. 50). Thus it was known that all cases of Addison's disease show a decrease in the absolute quantity of 11-oxygenated 17-ketosteroids or corticoidal 17-ketosteroids.

TABLE XXXV
Urinary 17 Ketosteroids

No.		Age	Sex	Time from onset to examination	Total 17-KS (mg/24 hrs)
1	Addison's disease	32	M	6 months	0.5
2	„	46	M	6 months	1.5
3	„	42	M	2 years	1.5
4	„	29	M	2 years	2.2
5	„	41	M	8 months	3.6
6	„	42	M	3 years	3.8
7	„	29	M	16 months	4.5
8	„	34	M	6 months	17.5
9	Riehl's melanosis	30	F	2 months	1.6
10	„	35	F	11 years	2.0
11	Lichen pigmentosus	46	F	1 year	1.7
12	„	30	M	6 months	2.4
13	Chloasma	42	F	4 months	7.4
14	„	39	F	2 years	8.2
15	„	34	F	6 months	9.6
16	Vitiligo	21	F		5.0
17	„	20	F		5.2
18	Albinism	20	F		6.1
Normal controls		4 cases	M		5.0～18.0
		5 cases	F		4.5～10.0

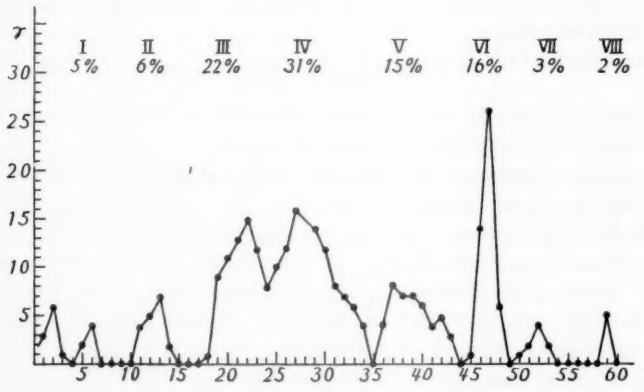

Fig. 49. Values of urinary 17-ketosteroid fractions in case 3, (man, 42 y.), of Addison's disease. Total 17-ketosteroids : 1.5 mg/day (297 γ).

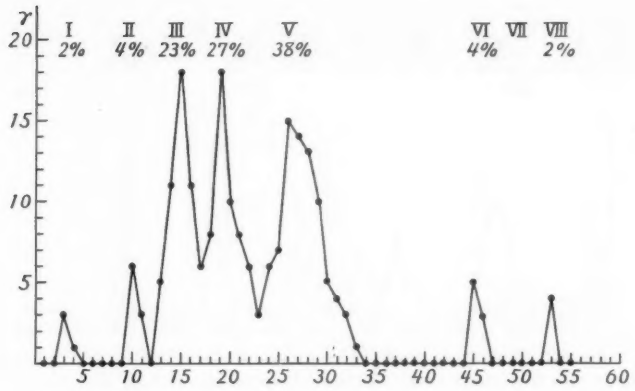

Fig. 50. Values of urinary 17-ketosteroid fractions in Case 8, (man, 34 y.), of slightly advanced Addison's disease. Total 17-ketosteroids: 17.5 mg/day (207 γ).

The amount of urinary 17-ketosteroids in Riehl's melanosis was estimated by Spira[11] to be decreased, and by Uno[14] to be approximately the physiological value in his 1 case. In our 2 cases, the total amount was decreased, but the rates of the fractions were almost normal (Fig. 51). In 2 cases of lichen pigmentosus, a decrease in 17-ketosteroids was observed as in Riehl's melanosis (Fig. 52). In chloasma, the total amount showed nearly normal values; but the glucocorticoidal 17-ketosteroids were found decreased in one of the 2 cases in which the fractions were measured (Figs. 53, 54).

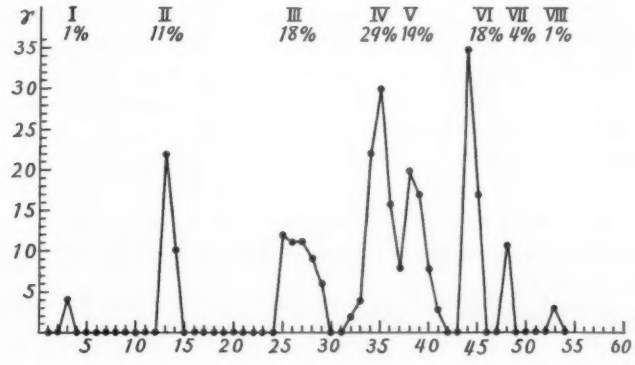

Fig. 51. Values of urinary 17-ketosteroid fractions in Case 9, (woman, 30 y.), of Riehl's melanosis. Total 17-ketosteroids: 1.6 mg/day (280 γ).

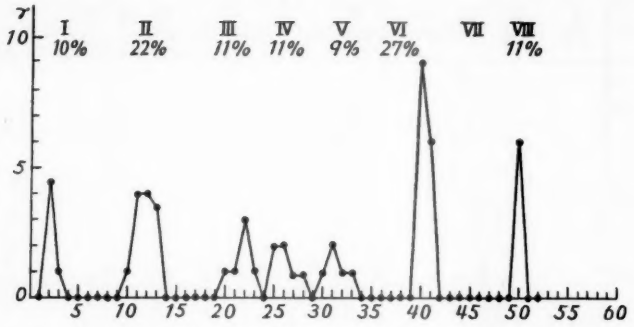

Fig. 52. Values of urinary 17-ketosteroid fractions in Case 11, (woman, 46 y.), of lichen pigmentosus. Total 17-ketosteroids: 1.7 mg/day (55.9 γ).

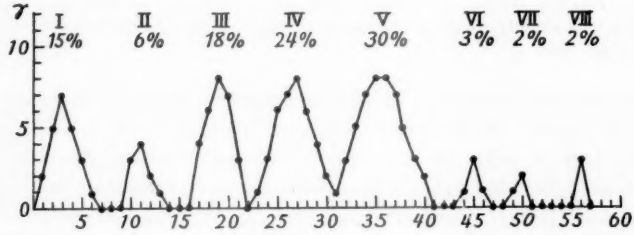

Fig. 53. Values of urinary 17-ketosteroid fractions in Case 13, (woman, 42 y.), of chloasma. Total 17-ketosteroids: 7.4 mg/day (158 γ).

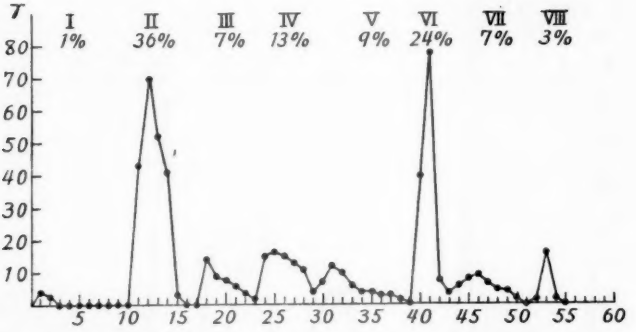

Fig. 54. Values of urinary 17-ketosteroid fractions in Case 15, (woman, 34 y.), of chloasma. Total 17-ketosteroids: 9.6 mg/day (575 γ).

In 2 cases of vitiligo, however, the 17-ketosteroids showed normal values, and in 1 case of complete albinism all the steroid-hormones showed

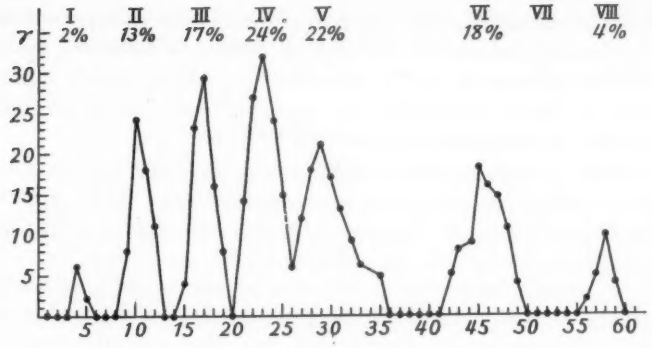

Fig. 55. Values of urinary 17-ketosteroid fractions in Case 18, (woman, 20 y.), of albinism. Total 17-ketosteroids: 6.1 mg/day (475 γ).

normal values (Fig. 55), suggesting that these diseases are of an obviously different mechanism, probably tyrosinase inactivation or denaturalizations in the epidermis.

To compare pregnants with cases of hyperpigmentation as regards chiefly 17-ketosteroids in the urine: in the former the androgenic fractions IV and V decrease with the progress of pregnancy and the corticoidal fractions VI and VII show a tendency to rise in the latter half period of pregnancy, but the total 17-ketosterioid content remains to be almost normal throughout the whole period as in chloasma; whereas in cases of hyperpigmentation, such as Addison's disease, Riehl's melanosis and lichen pigmentosus, the total 17-ketosteroid content is lowered in value, and the relative percentages of fractions IV and VII, being risen in no case, probably represent the actual decrease in their amount. Thus it seems that pregnant women and patients with hyperpigmentation are contrary to each other as regards the total urinary 17-ketosteroids, in particular, the quantities of Fractions VI and VII.

In short, the participation of the adrenocortical system in melanin formation can be partly conjectured from urinary 17-ketosteroids also.

Changes in Urinary Estrogen and Progesterone

As non-steroid hormones presumably related with skin pigmentation may be mentioned MSH, ACTH, gonadotropin, adrenalin and noradrenalin. ACTH expedites the secretion of glucocorticoids by acting upon the adrenocortex; besides, it has in itself a similar action as MSH, which is generally attributed to the probable coexistence of MSH[15),16),17)]. Gonadotropin acts upon the secretion of sex-hormones, while adrenalin

and noradrenalin control the action of MSH[16),18)]. Of these hormones, MSH has the strongest action on skin pigmentation as reported by many workers. Its administration to human bodies is followed by marked pigmentation (Lerner *et al.*[19)], Shizume[20)]). MSH, which increases in pregnancy, is highly responsible for hyperpigmentation in pregnants; it also increases in Addison's disease[16),20),21),22),23),24)].

To make a short mention of the recent literature on the relation of melanin formation to sex-hormones in particular: Davis *et al.*[25)], who administered estrogen to human subjects and guinea pigs, observed increased pigmentation on the nipples, halos and linea fusca but the absence of such pigmentation in menopausal women. According to Mighorst *et al.*[26)] and Rothman[27)], progesterone is said to expedite secretion of an MSH-like substance. Lerner *et al.* state that a small dose of it causes pigmentation in the skin of frogs but that no such phenomenon is caused by any of steroid hormones. Wheeler *et al.*[28)], however, have reported that local application of estrogen, but not of progesterone, causes pigmentation on the nipples of guinea pigs. According to Pfeifer *et al.*[29)], local application of testosterone causes pigmentation on the beak of a sparrow; and similar pigmentation occurs on the scrotum of a squirrel (Well[30)]) and in the skin of a castrated man (Edward *et al.*[31)]). According to Forbs[32)], the pellets implanted into the skin of male and female rats cause pigmentation in the hair, while Hamilton[7)] has reported on the pigmentation caused in tissue-cultivated skin by estrogen and androgen. Lerner and Fitzpatrick[15)], who have recognized no action of estrogen and androgen on melanin cells in the skin of frogs, have suggested that these hormones may have such an action in man.

As shown in these reports, estrogen and progesterone cause pigmentation in animal skin. But there have been hardly any reports dealing with the actual measurements of sex-hormone contents in patients with pigmentary skin diseases. For this reason we have made measurements of urinary estrogen and progesterone in pregnants and patients with chloasma, Riehl's melanosis, lichen pigmentosus and albinism, to study on what relationship there may be between these diseases and sex-hormones.

The experimental subjects were selected from among the patients treated at the Gynecology and Obsterics Clinic and the Dermatology Clinic of Tohoku University Hospital, and 24 hour urinary excretion was used as samples.

Urinary estrogen was estimated by Suzuki and Mori's method[33)]; and urinary pregnanediol, by Mack, Parks and McDonald's method with the help of the spectrophotometer (Hitachi)[34)]. These methods are both for chemical determination of the quantity of sex-steroids. Pregnanediol, however, is a product of progesterone metabolism, and has neither hormone

activity of its own, nor any relation to skin pigmentation (Lerner[16]), but was measured because an increase of this steroid in the urine is an index of increase of the progesterone in the body.

Experiments and Results

a. Pregnancy, parturition and puerperium

Fig. 56 shows the values of estrogen measured during normal pregnancy. Each point stands for the mean of values measured in 6–10 cases. The number of cases was 57 each for estrone, estradiol and estriol. In all cases, estrogen, in particular estriol, increased during pregnancy and decreased during puerperium.

Fig. 57 shows the results of 38 determinations of urinary pregnanediol. The values rise steeply with the course of pregnancy but fall suddenly on the 3rd day of puerperium, coming down to nearly nothing. The decrease of androgen and the increase of corticoids, as stated above in connection with the results of determinations of 17-ketosteroid fractions, are supposed to exert counteractions on increased pigmentation, but such actions seem to have been nullified by the marked increase of MSH, estrogen and progesterone during pregnancy.

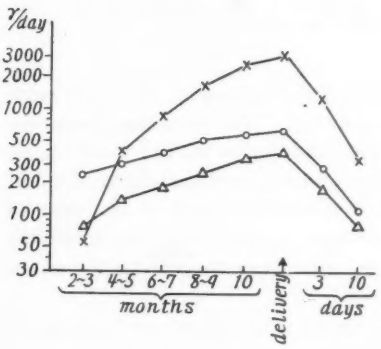

Fig. 56. Mean values of urinary estrogen in normal pregnancy, parturition and puerperium. —○— estron, —△— estradiol, —×— estriol.

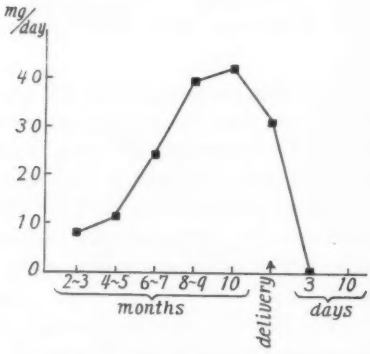

Fig. 57. Mean values of urinary pregnanediol in normal pregnancy, parturition and puerperium.

b. Changes in estrual hormones in chloasma and Riehl's melanosis

It has been pointed out by Crawford and Leeper[35], and others that urinary excretions of estrogen, progesterone and gonadotropin show definite changes according to the estrual stages. Fig. 58 shows the results

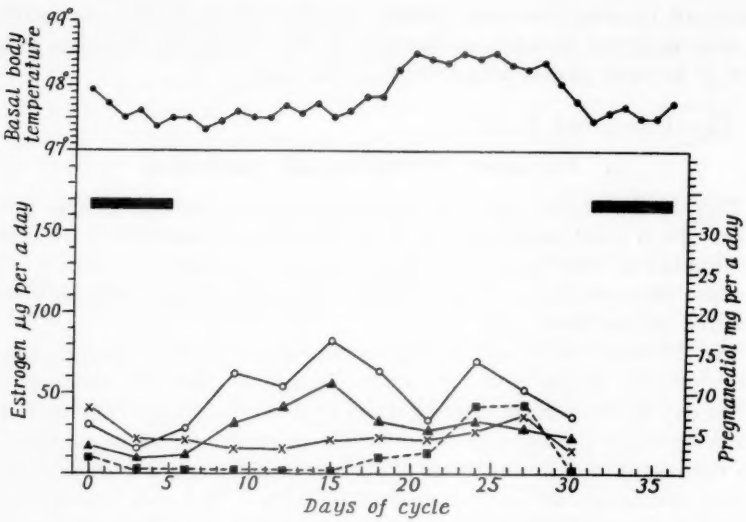

Fig. 58. Values of urinary estrogen and pregnanediol contents in a healthy woman, 31 y., with normal sexual cycle. ▬▬ Menstruation; —○— estrone, —▲— estradiol, —×— estriol, —■— pregnanediol. These marks will be applied also in following Figures (59–64).

of successive determinations in a normal adult woman of 31 years with the normal menstrual cycle, to serve as controls. Her basal temperature was of the double-phased type, and the estrone and estradiol values each showed a peak around the time of ovulation and the mid-stadium of luteal formation, respectively, each dropping to the lowest level at the time of menstruation. Pregnanediol amounts to little over zero in the follicular stage, increases in the yellow-body stage, and drops to zero in the menstrual stage.

Figs. 59–61 show the results of successive determinations throughout the menstrual cycle in 3 patients with chloasma. Two of them were adult women with the normal menstrual cycle, but the other had received uterectomy 2 years before and was amenorrheal, but her basal temperature was obviously of the double-phased type, lowering in the period before the ostensible ovulation time and rising after it, so that it was inferred that the endocrine function of sex-hormones was being performed quite normally in her. Thus, in all the 3 cases, estrone, estradiol and estriol each showed one peak in the ovulation stage and another in the middle of the luteal stage, while pregnanediol was found to fall almost to nothing before the ovulation but to rise after it. That is to say, the tendency of changes in

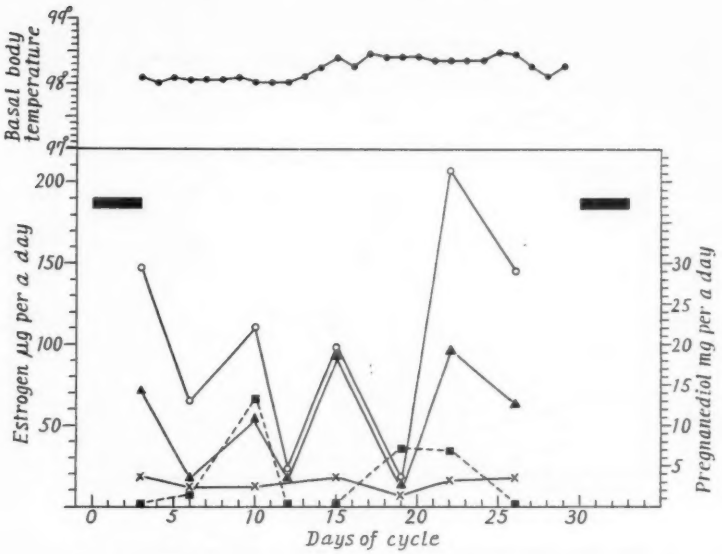

Fig. 59. Fluctuations of urinary estrogen and pregnanediol contents during a sexual cycle in a woman, 30 y., with chloamsa.

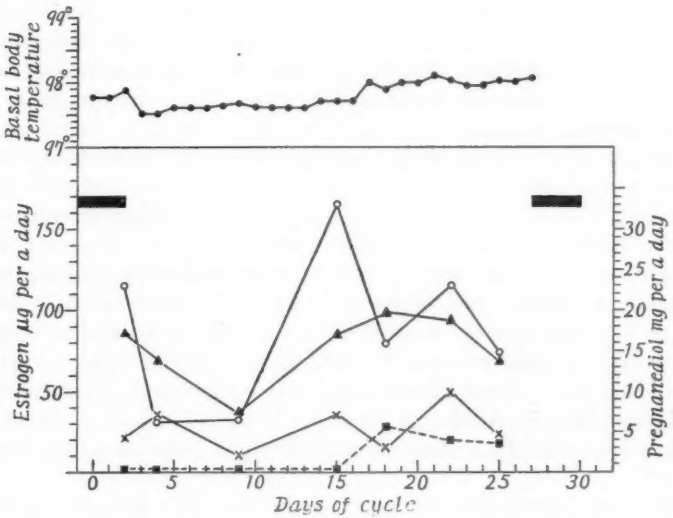

Fig. 60. Fluctuations of urinary estrogen and pregnanediol contents during a sexual cycle in a woman, 25 y., with chloasma.

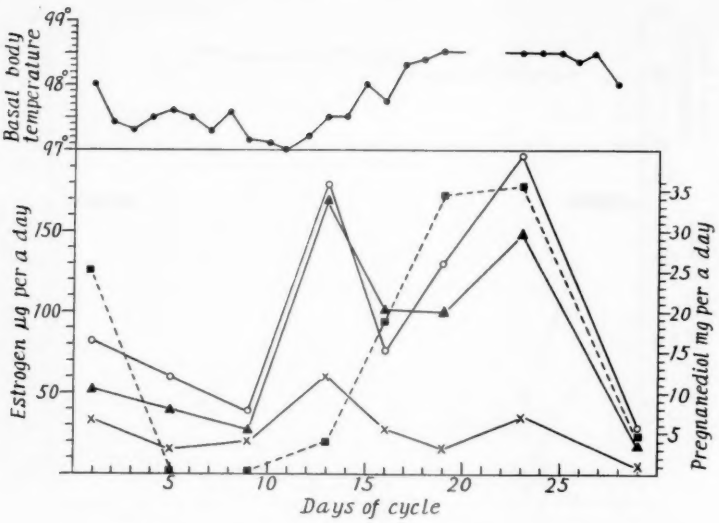

Fig. 61. Fluctuations of urinary estrogen and pregnanediol contents during a sexual cycle in a woman, 42 y., with chloamsa. (amenorrhoe due to uterectomy).

the estrual hormones in those cases was nearly the same as that in the control normal woman. The quantity, however, of these estrual female hormones, in particular of estrone, estradiol and pregnanediol was demonstrated to show markedly higher values in cases of chloasma than in the normal control.

Figs. 62, 63 show the similar changes of sex-hormones found in cases of Riehl's melanosis. The marked increase in estrone and estradiol observed in a woman (57 years) is a finding contradicting the observation by Davis cited above that no pigmentation followed estsogen administration to women in menopause and is to be noticed in studying the pathogenesis of Riehl's melanosis. We have also experienced a lowered basal metabolism and decrease of 17-ketosteroids in spite of normal menstruation in a female patient (Fig. 64) of 35 years suffering for 10 years from diffuse gray-brown pigmentation in her face somewhat resembling the pigmentation in Addison's disease or Riehl's melanosis, and surmised that it may be a pigmentary disease different from those alluded to in this case.

In short, we should not overlook the fact that in the comparative study of the estrual cyclic fluctuations of the urinary excretion of sex-hormones, a distinct increase was observed in the quantity of excreted

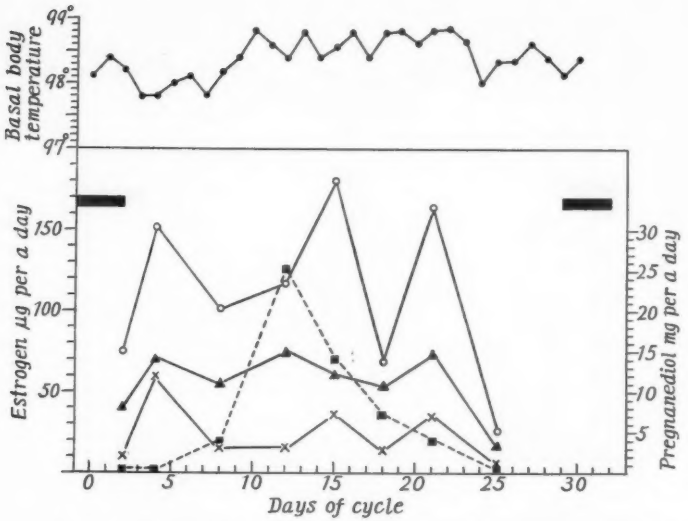

Fig. 62. Fluctuations of urinary estrogen and pregnanediol contents during a sexual cycle in a woman, 46 y., with Riehl's melanosis.

hormones in cases of chloasma and Riehl's melanosis than in a normal woman.

Besides, as shown in the preceding chapter, the chloasma cases showed normal values of urinary 17-ketosteroids, while in the cases of Riehl's melanosis the value was lowered, indicating a difference in the function of the adrenocortical system between the two diseases. This might give a hint on the pathogenesis of hyperpigmentation on the face in the two diseases.

Furthermore, the merely temporary increase in urinary 17-ketosteroid values at the time of parturition, and the gradual increase of estrogen and pregnanediol during pregnancy despite the decrease of Fractions IV and V of 17-ketosteroids originating in androgen, in combination with the increase of MSH during pregnancy, as reported in literature, seem to be the cause of pigmentation during pregnancy and give an interpretation of clinical observations of the localities of pigmentation different from those in pigmentary diseases. These relationships may be illustrated in the following Table XXXVI which will show the different tendencies there are between them.

Summary

We have made measurements of steroid hormones in urine sampled

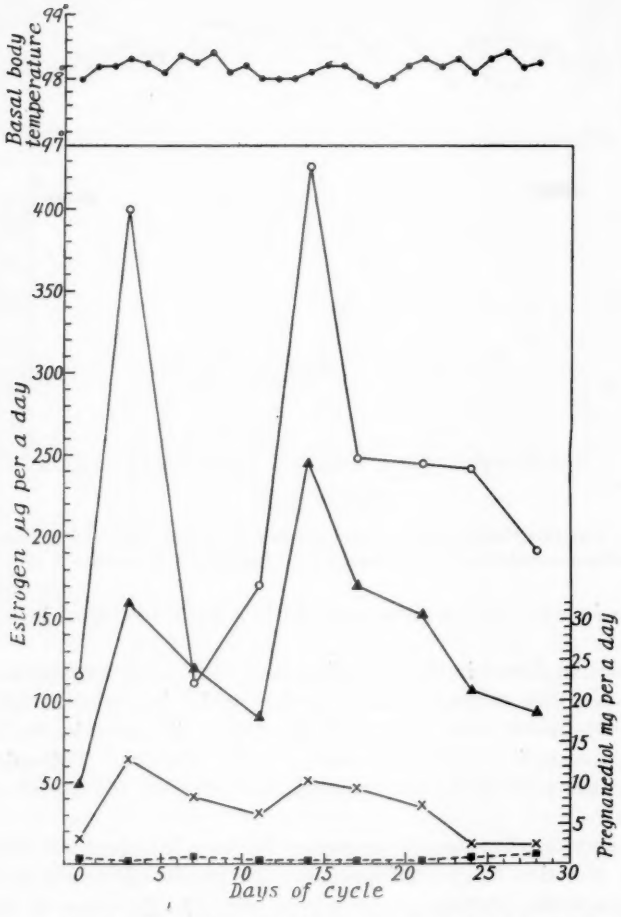

Fig. 63. Fluctuations of urinary estrogen and pregnanediol contents during a sexual cycle in a woman, 57 y., with Riehl's melanosis (menopause).

from subjects showing increase of skin pigmentation, including women in pregnancy, parturition, and puerperium and patients with pigmentary skin diseases, by chemical methods and arrived at the following results:

1. The values of 11-oxygenated 17-ketosteroids were lowered in cases of Addison's disease, chloasma, Riehl's melanosis and lichen pigmentosus.

2. Estrogen and pregnanediol were found to be increased in preg-

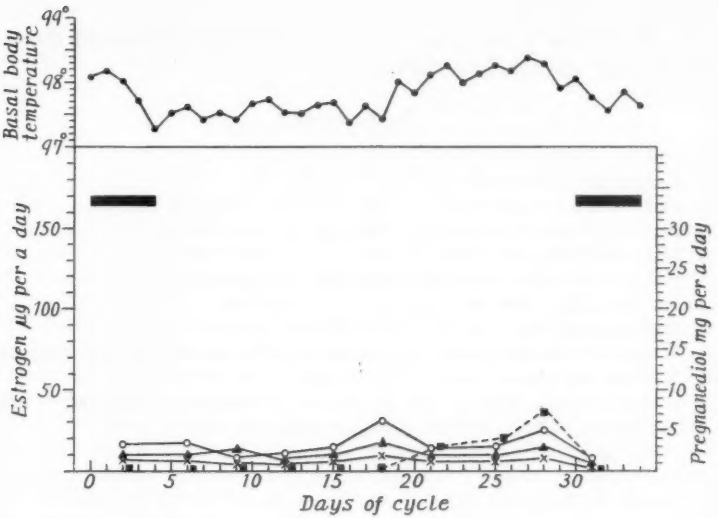

Fig. 64. Fluctuations of urinary estrogen and pregnanediol contents during a sexual cycle in a woman, 35 y., with a type of chronic diffuse melanosis on the face.

TABLE XXXVI

	Pregnancy	Chloasma (non-pregnant)	Riehl's melanosis (non-pregnant)
Pigmented Sites	Nipples, haloes, linea fusca, external genitals	Face	Face
17-KS: Total	Increased only at delivery	No change	Decreased
17-KS: Fractions	Decreased IV, V	Decreased VI, VII	Normal rates
Estrogen	Iecreased (espec. estriol)	Increased by menstrual cycle	Increased by menstrual cycle
Pregnanediol	Increased	Increased by menstrual cycle	Increased by menstrual cycle

nants and in patients with chloasma and Riehl's melanosis, the increment being much higher in the former than in the two latter diseases. The increases of contents of these hormones at the menstrual cycles tended to be higher in female patients of chloasma and Riehl's melanosis than in a normal non-pregnant woman.

3. Consequently, these hormones are presumed to participate in melanin formation during pregnancy and in the diseases alluded to in

Conclusions 1 and 2.

4. No anomaly was observed in the urinary contents of these hormones in cases of complete albinism.

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APPENDIX

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Tohoku J. Exp. Med., 1955, **55**, Supplement I.

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XXI. Malignant Melanoma in Japan
Tohoku J. Exp. Med., 1953, **57**, 385.

XXII. Nevus Fusco-caeruleus Acromio-deltoideus
ibid., 1954, **60**, 10.

General Conclusion of Report I

In summarizing our experiments mentioned in the foregoing chapters, the following conclusions can be reasonably reached.

The Participation of Nervous System in Melanin Production

By the results of Bielschowsky-Seto's silver impregnation method, it is found that nevus cell of pigmented nevus originated from neuroectodermal system which is homogenetical to Schwannian cells just as Masson stated. In an autopsy case of abortive Recklinghausen's diesease without neurofibroma, preneurinomatous changes were noticed microscopically in the pigmented area and in solar plexus. Further circumscribed increase of Schwannian nuclei of larger nerve bundle is observed in the deeper dermal layer of pigmented area. These findings disclose the fact that there exists close relationship between Recklinghausen's fleck and neurofibroma, and are influential data of nerve cell theory stated by Verocay, Held, Kyrle, Masson, Cailliau, Jausion-Levy-Coblentz-Spillmann and recently by John and Ormea. The frequent occurrence of socalled neurofibromatous structure of nevus cell is reported by Becker who in the histological study of 741 cases of pigmented nevi found in 72.5% the structure of the third type of Miescher. Thus the nevus cell which may present polymorphism according to the local peculiarities, is uniformly of neuroectodermal origin. These facts give clue also to the polymorphic histological picture of melanocarcinoma.

On the contrary, the pigment cell of Mongolian spot, blue nevus and nevus fusco-caeruleus belong to mesenchymal melanoblast. In the latter two diseases which are properly interpreted as localized malformation, the histological structure is apparently more exaggerated than in physiological character *i.e.* Mongolian spot, and the affinity to nervous system is more conspicuous suggesting the coincidence with endo- or perineurium. This finding as well as the results of phylogenetical investigation of Mongolian spot, leads us to the conclusion that the pigment cell in these dermatoses belong to homogenetical melanoblasts in the sense of perineurale Pigmenthülle of Weidenreich. Though Mongolian spot disappears ontogenetically in a prescribed duration in which it becomes estranged itself from nervous system and on the contrary, blue nevus and nevus fusco-caeruleus are interpreted as the localized malformation close to nervous system and exist for life. This reminds one of the importance of correlation between melanin formation and nervous system. Thus we were able to verify both histologically and genetically that both ectodermal and mesodermal melanoblasts are closely related to nervous system. The conception of mesectoderm of Platt which was introduced into the genetical experiment on the mechanism of development of pigment cell and pigment flecks in amphibia by Harrison, Holtfreter, Du Shane, Twitty, Rosin, De Lanney and Tada is of interest in this connection.

In many clinical cases of systematized nevi, incontinentia pigmenti, nevus ophthalmomaxillaris Ota and Mongolian spot, the eruption are localized in such a manner as one can easily assume the coincidence with the distribution of cutaneous nerve. Such cases are reported in the corresponding chapters. In various pigment disorders, functional abnormalities of vegetative nervous system are disclosed by the results of histamine scratch test and diaphoretic test. In some cases of pigmented nevus and incontinentia pigmenti, the abnormality of trophic nerve distribution resulted in the asymmetry of the breast glands. In several cases of nevus spilus en nappe, even the sensible nerve was involved in the affection. These are homolateral nervous disturbances accompanying pigmentary disorders and are favorable to our theory.

In benign melanomas, I believe, it is possible to distinguish rather distinctly those originating

from Schwannian cellular ectodermal melanoblasts and those from mesenchymal melanoblasts corresponding to endo- or perineurium. So it is theoretically not impossible to differentiate histologically melanosarcoma and melanocarcinoma. This differentiation, however, is not always possible in individual cases. We believe that this problem well be given a solution in further progress in the investigation.

The Rôle of Enzymes in Melanin Production

The conflict of the monism and dualism on this problem was already mentioned. We tried indophenolase reaction in the melanoblasts which were considered as dopa positive by many investigators such as cells of basal layer, pigmented nevus, Mongolian spot, blue nevus, nevus fusco-caeruleus and malignant melanoma or pigment cell of Riehl's melanosis and found numerous indophenolblue granules in their protoplasm. Their size and form coincide quite well with those of preexistent melanin granules. And they are more numerous in cells with little melanin content. While on the other hand positive peroxidase reaction is principally limited to nuclei, and the reaction is especially strongly positive in the nuclei of basal cells, nevus cells and dermal melanoblasts, where diffuse or granular staining of protoplasm is also noticed. Endothel cells of blood capillaries present strong positive reactions, and indophenolblue granules and peroxidase positive granules overflow to the surrounding tissue. And at the same time local blood leucocyte of pigmented area shows stronger peroxidase reaction than normal area. This shows that there occurs exchange of both enzymes between myeloic leucocytes and tissue cells. These biochemical reaction exhibit the passing phase of melanin production in the presence of two enzymes, oxidase and catalase in the protoplasm. In permanganate methylgreen staining of Unna the site of melanogenesis stains always with methylgreen as oxygen areas. And in vitiligo the papillary layer reduces permanganate as reduction areas. In melanoma amelanotic foci also reduce permanganate, like nevus cell nest without melanin. Thus the lack of melanogenesis in malignant melanoma is no less than a phase of extremely rapidly metastatic and infiltrative growth of tumor cells and cannot be estimated as a measure of malignancy. This finding coincides with the experimental results of Greenstein on the oxygen consumption of melanotic and amelanotic mouse melanoma suspension.

It is well known that malignant melanoma is rare in Negroes and that it frequently occurs in area which is physiologically poor in pigment such as finger tip, sole and lip. So it may be properly assumed that the disturbance of biochemical condition resulting in abnormal melanin production in heterotopic area is a cause of malignancy like the incidence of malignant melanoma in white horse.

In our follow-up study of Urbach-Kral's experiment, the increase of oxygen area is noticed in area hyperpigmented by the application of bergamot oil. The injection of ascorbic acid and riboflavin prior to the application of bergamot oil and ultraviolet irradiation completely inhibits hyperpigmentation. On histochemical examination the increase of reduction area is noticed. By injection of these vitamins the cutaneous penetration of bergamot oil is reduced and the decomposition product of this substance reacts rather reductively on the skin resulting in the suppression of melanin production. In Riehl's melanosis the deficiency of vitamin C and B complex is surmized by the increased values of pyruvic acid and iodic acid reducing substance of blood serum. These vitamin deficiencies are important etiological factors in Riehl's melanosis. The abovementioned results of Unna's staining, indophenol oxidase reaction and peroxidase reaction point to the importance of local biochemical condition specially of redox-potential and the action of biocatalyst in normal and pathological melanogenesis.

In the experiment with black goldfish, only one of the three isomers of dioxybenzenes, namely hydroquinone can suppress melanin production. This action is attributed to the decreased alkalization potency of cutaneous tissue by hydroquinone. Recent observation of Dejust, Ropshaw, Cailliau on the dermoepidermal hydrogen ion concentration supplies further

influential data to this theory.

On the other hand, by injection of *p*-benzoquinone to yellow-red goldfish, black pigment is formed and the dopa reaction of scale turned to positive. This newly formed pigment is identified as melanin. As all other oxidizing agents failed to give similar results, it is not a mere oxidation by *p*-quinone but a special catalytic action of this substance on melanogenesis.

That the enzyme tyrosinase is indispensable for melanogenesis is admitted by almost all investigators. This ferment is actually identified in the extract of human and animal melanosarcoma by Niklas. In normal human tissue, however, it has been impossible to obtain tyrosinase activity. This may be due to the presence of inhibitor substance like ascorbic acid and sulfhydryl compounds (Hoff). While on the other hand Bloch and Raper emphasized the participation of dopa oxidase. And in my experiment indophenolase and peroxidase are observed in the histochemical study, so their participation in melanogenesis is readily surmised. Thus I believe the two enzymes theory is more probable. Then how is the action of *p*-quinone in the goldfish experiment explained? According to Mann and Keilin in the presence of small amount of *o*-quinone polyphenolase catalyzes not only polyphenol but also monophenol. *p*-Benzoquinone may act in the same way as an activator and thus accerelate melanogenesis. Both indophenoloxidase and peroxidase have common substrate and their difference lies only in acceptor substance. So their activities are presented at various passing phases of biochemical condition of individual tissue cell. The manifestation of dopa oxidase activity represents a phase of this cellular activity and cannot always be estimated as the existence of specific enzyme. This pigment production in goldfish appears first and most prominently at the place where carotinoid pigment is preexistent. And the addition of vitamin A, B_2 and C to *p*-quinone accelerates melanin production. So the participation of cytochrom system cannot be overlooked.

From the above histological and histochemical researches, I have concluded that melanogenesis is a part of biological oxidizing processes which is carried on under the influence of nervous system. In this sense the chemical transmission theory of Loewi-Dale and/or neurohormonal theory of Vilter is introduced in the mechanism of melanogenesis.

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